Calmodulin-dependent protein kinase gamma 3 (CamKIIγ3) mediates the cell cycle resumption of metaphase II eggs in mouse

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Mature mammalian eggs are ovulated arrested at meiotic metaphase II. Sperm break this arrest by an oscillatory Ca2+ signal that is necessary and sufficient for the two immediate events of egg activation: cell cycle resumption and cortical granule release. Previous work has suggested that cell cycle resumption, but not cortical granule release, is mediated by calmodulin-dependent protein kinase II (CamKII). Here we find that mouse eggs contain detectable levels of only one CamKII isoform, gamma 3. Antisense morpholino knockdown of CamKIIγ3 during oocyte maturation produces metaphase II eggs that are insensitive to parthenogenetic activation by Ca2+ stimulation and insemination. The effect is specific to this morpholino, as a 5-base-mismatch morpholino is without effect, and is rescued by CamKIIγ3 or constitutively active CamKII cRNAs. Although CamKII-morpholino-treated eggs fail to exit metaphase II arrest, cortical granule exocytosis is not blocked. Therefore, CamKIIγ3 plays a necessary and sufficient role in transducing the oscillatory Ca2+ signal into cell cycle resumption, but not into cortical granule release.

KEY WORDS: Calcium, Cell cycle, Fertilization, Mouse, CamKII (Camk2)

INTRODUCTION

Mouse eggs are ovulated following arrest at metaphase of the second meiotic division (metII). Fertilization breaks this arrest, with the egg extruding a second polar body (PB2) and forming pronuclei. Ca2+ spikes induced by phospholipase C zeta, which are introduced into the egg on gamete fusion, are responsible for causing the degradation of Erp1/Emi2 (Fbxo43 – Mouse Genome Informatics) (Ducibella and Fissore, 2008; Jones, 2005; Mehlmann, 2005; Swann et al., 2006). Erp1/Emi2 loss activates the anaphase-promoting complex (APC) and so drives exit from meiosis (Madgwick et al., 2006; Shoji et al., 2006).

In frog eggs, the Ca2+ fertilization signal switches on calmodulin-dependent protein kinase II (CamKII; Camk2), which phosphorylates Erp1/Emi2 and so promotes its degradation (Liu and Maller, 2005; Rauh et al., 2005; Schmidt et al., 2005). Consistent with this more recent development in the understanding of the molecular events of activation, it had been discovered several years previously, also in frog eggs, that a constitutively active CamKII (CA-CamKII) is able to recapitulate the events of cell cycle resumption (Lorca et al., 1993; Morin et al., 1994). The ability of CA-CamKII to induce cell cycle resumption was recently reiterated in intact mouse eggs (Madgwick et al., 2005), in keeping with a CA-CamKII to induce cell cycle resumption was recently repeated (Lorca et al., 1993; Morin et al., 1994). The ability of CA-CamKII to induce cell cycle resumption was recently repeated in intact mouse eggs (Madgwick et al., 2005), in keeping with a conserved mechanism of Ca2+-activated signal transduction in vertebrate species. Further support for a role of CamKII in meiotic resumption has come from the measurement of rises in endogenous CamKII activity in mouse eggs during Ca2+-induced activation (Markoukali et al., 2003; Markoukali et al., 2004).

Despite the importance of CamKII in transducing the Ca2+ signal in the cell cycle resumption of eggs, two questions as to its role remain unresolved. Firstly, which CamKII isoform transduces the Ca2+ signal? CamKII exists as a family of proteins, with α, β, γ and δ subgroups (Hudmon and Schulman, 2002a; Hudmon and Schulman, 2002b). Cells often express more than one isoform, and their effects can be isoform specific (Backs et al., 2009; Shen et al., 1998; Walikonis et al., 2001). Secondly, do redundant Ca2+ signaling pathways operate at fertilization to ensure cell cycle resumption? In recent years, other kinases have been reported to cause cell cycle resumption in mammalian eggs, notably protein kinase C, but, critically, its involvement has always been questionable, relying on the specificity of pharmacological inhibitors and activators to support such a role (Ducibella and Fissore, 2008; Jones, 1998). In the present study, we examined the CamKII profile of mouse eggs with a view to using a targeted antisense knockdown approach to determine which CamKII isoform is responsible for cell cycle resumption, and also to determine whether any CamKII-independent pathway could be switched on by a Ca2+ signal in eggs to similarly induce cell cycle resumption.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma-Aldrich unless otherwise specified.

Oocyte collection and incubation medium

Germinal vesicle (GV) oocytes from 3- to 4-week-old C57Bl6 mice were collected and denuded in M2 medium, 44-50 hours after injection of 10 IU pregnant mare’s serum gonadotrophin (Intervet). For long-term incubation, oocytes were cultured in MEM with 20% fetal calf serum (Invitrogen) at 37°C in 5% CO2 (Reis et al., 2006). Milrinone (1 μM) was used to arrest GV oocytes. For parthenogenesis, oocytes were cultured in M2 medium with Ca2+ replaced by 3 mM Sr2+ (Bos-Mikich et al., 1997) or in M2 medium with 7% ethanol (Cuthbertson et al., 1981).

cDNA library and PCR

cDNA was prepared from 1350 oocytes using the Smart PCR cDNA Synthesis Kit (Clontech) according to the manufacturer’s instructions. cDNA was used for PCR and amplified with Hot Start DNA Polymerase (Invitrogen); annealing temperatures were from 50-65°C. PCR primers used for the different CamKII isoforms were (Fwd, forward; Rev, reverse; 5’-3’): α1, Fwd ATGCCAATTGCGCATGCTGCTTTTCTACCG; β1, Fwd ATGCAGATCGACATCGCCG; β2, Fwd ATGCAGATCGCCATGCGCCAGAG; α2, Fwd ATTCGATTGCGCATTACATTCACCTGC and Rev GATCGTGCACATGCGGGCAGGG; β, Fwd ATGCAGATCGCCATGCGCCACCCAGGT

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and Rev GATCGTGACCTGCAGCGGCCGAC - TTGCCG; δ1-3 Fwd ATCGCGAATTCCGAGACCTGCCGACCCA - δ1 Rev GATCGTGACCTGCAGCGGCCGAC; δ2 Rev GATCGTGACCTGCAGCGGCCGAC - TTGCCG; δ3 Rev GATCGTGACCTGCAGCGGCCGAC. Ethidium bromide-stained gel fragments were excised and purified prior to TA ligation into the pGEM T-Easy vector or pRN3 vector for sequencing. CamKIIγ3 cRNA was prepared using a modified pRN3 vector (Reis et al., 2006). cRNA was synthesized as described previously (Chang et al., 2004). CamKIIγ antisense morpholino (CamKII-MO) 5'-ACGTCGGTGCACAGTCACCGCCGCCC-3' (Gene Tools LLC), and a 5-base-mismatch morpholino (CamKII-5MM-MO) 5'-ACCTTCGTCGACTGCACCGCCGCCC-3', were used at 1.5 mM.

Cortical granule exocytosis

Eggs were incubated for 30 minutes following ethanol activation and then fixed with 3.7% paraformaldehyde, then washed with PBS containing 1% polyvinylpyrrolidone (PBS/PVP) extensively prior to incubation with FITC-conjugated Lens culinaris lectin at 10 μg/ml (EY Laboratories, San Mateo, CA, USA).

Microinjection and imaging

Microinjections were performed as described previously (Nixon et al., 2002; Reis et al., 2007). For intracellular Ca2+ measurement, oocytes were incubated for 30 minutes with 5 μM fluo-4-AM in M2 (Invitrogen) and 0.01% pluronic F127 (Calbiochem). Intracellular imaging and in vitro fertilization were as described previously (Nixon et al., 2002). Brightfield and epifluorescence images were recorded using a Princeton Interline MicroMax CCD camera. MetaMorph and MetaFluor software (Molecular Devices, Downingtown, PA, USA) were used for image capture and data analysis.

Immunofluorescence

Immunofluorescence was performed on fixed and permeabilized oocytes as described previously (Reis et al., 2006). Oocytes were incubated with antibodies for tubulin (1:200; A11126, Molecular Probes) or CamKIIγ (1:50; 12666, Protein Tech, Chicago, IL, USA). Detection was with FITC (1:100; F0270, Dako) or TRITC (1:100; R0156, Dako) conjugated secondary antibodies. For chromatin staining, oocytes were incubated with 10 μg/ml Hoechst 33258 for 10 minutes. Oocytes were mounted in SlowFade (Invitrogen). Images were acquired using an Olympus FV1000 confocal microscope equipped with a 60×/1.2 NA UPLSAPO oil-immersion objective.

RESULTS AND DISCUSSION

CA-CamKII induces meiotic resumption in mouse eggs (Knot et al., 2006; Madgwick et al., 2005). CamKII is therefore thought to be the downstream kinase activated by the sperm Ca2+ signal at fertilization. Here, we sought to determine which CamKII family members are present in eggs, and to determine the effects of their knockdown on cell cycle resumption following meI arrest.

Eggs express only the CamKIIγ3 isoform

Mammalian cells contain four CamKII gene family members, the pre-mRNAs of which are often spliced to generate a large number of CamKII variants. We used a PCR-based strategy to examine the CamKII profile in eggs and found only CamKIIγ1 (Fig. 1), consistent with previous EST screens of mouse eggs (Eviskov et al., 2006). The ability of each primer pair to produce a PCR product of the expected size was confirmed under identical PCR conditions using brain (α, β, δ) or whole ovary (γ) as positive controls (Fig. 1). CamKIIγ has three splice variants (see Fig. S1 in the supplementary material). The primers used cannot discriminate between these, so to determine which splice variant is most prevalent we transformed the PCR product into a pRN3 vector, transfected E. coli cells, and sequenced isolated plasmids from ten random clones. All clones were found to be CamKIIγ3 (see Fig. S2 in the supplementary material). Therefore, we conclude that mouse eggs express predominantly CamKIIγ3, and if other CamKII proteins are present then they are likely to be other splice variants of CamKIIγ and present in much lower abundance than CamKIIγ3.

CamKIIγ knockdown does not inhibit oocyte maturation

Our strategy was to knockdown CamKIIγ3 expression in mature eggs using an antisense morpholino (MO). We injected CamKII-MO into germlinal vesicle (GV) oocytes that were allowed to mature spontaneously in culture. Overall, microinjection procedures at the GV stage mildly impaired maturation rates, but this effect was non-specific and was also observed with a 5-base-mismatch MO to CamKII (CamKII-5MM-MO), as well as with buffer alone (see Fig. S3 in the supplementary material). Similarly, we did not observe any improvement in maturation rates for CamKII-MO-injected oocytes that were co-injected with CamKIIγ3 cRNA. Such ‘rescue’ experiments were made possible by the absence of the MO-targeted 5'-UTR in the cRNA construct. The eggs injected with CamKII-MO that fully matured appeared indistinguishable from non-injected controls (see Fig. S3 in the supplementary material).

We have previously demonstrated the efficacy of MO knockdown using immunoblotting (Reis et al., 2006; Madgwick et al., 2006). However, the anti-CamKIIγ antibody employed here was insufficiently sensitive considering the small numbers of oocytes that can be gathered following microinjection. Instead, we used an immunofluorescence-based approach on individual fixed and permeabilized oocytes to (1) confirm the ability of the CamKII-MO to knockdown CamKII expression; (2) demonstrate the lack of effect of the control CamKII-5MM-MO; and (3) confirm the expression of CamKII cRNA in CamKII-MO-treated oocytes (Fig. 2).

CamKIIγ knockdown inhibits meiotic exit in meiosis II

The above data suggest that CamKII-MO-injected eggs that had extruded a first polar body (PB1) were phenotypically normal. However, such eggs showed poor responses to the Ca2+ mimetic Sr2+, which, like sperm, induces an oscillatory activation signal.
are capable of extruding a PB2, albeit very slowly. Completely block meiotic resumption, and consequently these eggs CamKII-MO-injected eggs, the level of knockdown is insufficient to (Fig. 3C). The simplest interpretation of these data is that in some extrude a PB2 (~20%, Fig. 3A) did so over a much longer timecourse control eggs, those injected with CamKII-MO that managed to parthenogenetic activation (Chang et al., 2004). In contrast to these expected, as PB2 extrusion is known to occur at this time following CamKII-MO, but not 5MM-MO, treatment, as well as the ability of the CamKII cRNA to raise CamKII levels. The number of oocytes used is shown in parentheses (pooled from two to three independent experiments). Error bars indicate s.d., *, P<0.05 (Student’s t-test).

(Bos-Mikich et al., 1997; Kline and Kline, 1992; Madgwick et al., 2006). Uninjected, in vitro matured (IVM) eggs, or those that had been injected with CamKII-5MM-MO, gave high rates (~80%) of PB2 extrusion and pronucleus formation, the morphological manifestations of meiotic completion (Fig. 3A). By contrast, the vast majority of IVM eggs that had been injected with CamKII-MO failed to extrude a PB2 or form pronuclei, but instead remained arrested at metaphase (Fig. 3A,B).

In eggs microinjected with CamKII-MO that managed to extrude a PB2, the timing of PB2 extrusion was very much delayed. In Fig. 3A we present polar body extrusion rates 8 hours after Sr2+ stimulation. In IVM eggs that were not injected at the GV, or those that were injected with the control CamKII-5MM-MO, the PB2 was extruded ~2 hours after Sr2+ stimulation. These timings are as expected, as PB2 extrusion is known to occur at this time following parthenogenetic activation (Chang et al., 2004). In contrast to these control eggs, those injected with CamKII-MO that managed to extrude a PB2 (~20%, Fig. 3A) did so over a much longer timecourse (Fig. 3C). The simplest interpretation of these data is that in some CamKII-MO-injected eggs, the level of knockdown is insufficient to completely block meiotic resumption, and consequently these eggs are capable of extruding a PB2, albeit very slowly.

The lack of meiotic resumption was not due to off-target inhibition of Ca2+ signaling by the MO, nor to any essential role of CamKII in Ca2+ release, as the Ca2+ responses appeared similar in CamKII-MO-injected and CamKII-5MM-MO-injected eggs (Fig. 4). In addition, the effect of the MO knockdown appeared specific as it could be rescued. To perform a rescue experiment, either CamKIIγ3 cRNA was injected together with CamKII-MO at the GV stage, or CA-CamKII cRNA was injected at the meiI stage, and both approaches rescued sensitivity to the Sr2+-containing medium (Fig. 3A). Importantly, these two rescue experiments ruled out the possibility that IVM eggs produced following CamKII-MO injection were actually arrested at meiI because of a spindle assembly checkpoint. This is because meiI arrest by activation of the spindle assembly checkpoint is not Ca2+ sensitive, whereas physiological, cytostatic factor-mediated arrest manifestly is (Jones, 2005; Jones et al., 1995; Tsurumi et al., 2004).

Eggs that had been in vitro matured following CamKII-MO injection and were then inseminated showed an oscillatory Ca2+ signal, but failed to show signs of meiotic resumption (Fig. 4). These observations are therefore the same as those made with the parthenogenetic agent Sr2+. This would be predicted given that Ca2+ is the necessary and sufficient trigger provided by sperm at
CamKIIy knockdown does not inhibit cortical granule release

Although the CA-CamKII construct induces cell cycle resumption, it has very little effect on cortical granule (CG) release (Knott et al., 2006), probably because CG release is CamKII independent and initiated by myosin light chain kinase (Matson et al., 2006). However, we thought it interesting to confirm the lack of primary involvement of CamKII in CG release. IVM eggs that were arrested at the metaphase stage were activated using 7% ethanol. This would enable us to measure CG release within a narrow time window following a stimulation that would be both synchronous and achieve good rates of release. Oscillatory spiking activity in eggs following Sr2+ addition can be variable (Madgwick et al., 2004). By contrast, a Ca2+ rise is initiated immediately upon ethanol addition (Cuthbertson et al., 1981) and ethanol-induced CG release is equivalent to that induced by sperm (Kim and Schuetz, 1993). Following fixing and lectin staining at 30 minutes post-ethanol treatment, we observed that all ethanol-treated eggs, regardless of CamKII-MO injection, showed a cortical ring of exudate staining (see Fig. S4 in the supplementary material). These data therefore complement those obtained with CA-CamKII, and suggest that CamKII is not the primary transducer of CG release.

Previously, CamKII activity has been measured with each Ca2+ spike that is associated with the sperm oscillatory activating signal (Markoulaki et al., 2003; Markoulaki et al., 2004), and its constitutive activity induces meiotic resumption (Knott et al., 2006; Madgwick et al., 2005). The present findings therefore present the third component in proving the requirement of a protein activity for a physiological process, these being to (1) measure that activity during the process; (2) mimic the process by activating the protein, and (3) inhibit the process by blocking its activity. In conclusion, the present data offer support for the hypothesis that CamKIIy acts by itself to induce cell cycle resumption in mouse eggs at fertilization.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/24/4077/DC1

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Camk2g_1  MATTATCTRFTTDDYQLFEELGKAFSVVRRCVKKTSTQGAYAIKINTKKSARDHQKLER 60
Camk2g_2  MATTATCTRFTTDDYQLFEELGKAFSVVRRCVKKTSTQGAYAIKINTKKSARDHQKLER 60
Camk2g_3  MATTATCTRFTTDDYQLFEELGKAFSVVRRCVKKTSTQGAYAIKINTKKSARDHQKLER 60

Camk2g_1  EARICRLKHNPVRHLDSEIEEGHYLVFDLVTGEGELFEDIVAREYSEADASHCIQI 120
Camk2g_2  EARICRLKHNPVRHLDSEIEEGHYLVFDLVTGEGELFEDIVAREYSEADASHCIQI 120
Camk2g_3  EARICRLKHNPVRHLDSEIEEGHYLVFDLVTGEGELFEDIVAREYSEADASHCIQI 120

Camk2g_1  LESNVHNIHQDHVRDLKENLASSCIGAVLADGAYLVEVQGEQQAWFGFATG 180
Camk2g_2  LESNVHNIHQDHVRDLKENLASSCIGAVLADGAYLVEVQGEQQAWFGFATG 180
Camk2g_3  LESNVHNIHQDHVRDLKENLASSCIGAVLADGAYLVEVQGEQQAWFGFATG 180

Camk2g_1  LSPEVLKDPYKYKLVDMACGVLILVGLSYFPPFDQHKLQIKAGAYDFPSPWDT 240
Camk2g_2  LSPEVLKDPYKYKLVDMACGVLILVGLSYFPPFDQHKLQIKAGAYDFPSPWDT 240
Camk2g_3  LSPEVLKDPYKYKLVDMACGVLILVGLSYFPPFDQHKLQIKAGAYDFPSPWDT 240

Camk2g_1  VTPEAKNLMQNLNPKRDQALKHNPVCRSTSVSMMHRQETVECLRFKRNARKKL 300
Camk2g_2  VTPEAKNLMQNLNPKRDQALKHNPVCRSTSVSMMHRQETVECLRFKRNARKKL 300
Camk2g_3  VTPEAKNLMQNLNPKRDQALKHNPVCRSTSVSMMHRQETVECLRFKRNARKKL 300

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Camk2g_2  KGAILTTMLVSRNFSAAKSLNKKSDGGVK---------------------PSQSNLVSQPEPAPL 349
Camk2g_3  KGAILTTMLVSRNFSAAKSLNKKSDGGVK---------------------PSQSNLVSQPEPAPL 330

Camk2g_1  QTAMEPQTTVHNAERDGKSTESCNTETTEDKLRQIEKITEQLEIAINGDFEAY 420
Camk2g_2  QTAMEPQTTVHNAERDGKSTESCNTETTEDKLRQIEKITEQLEIAINGDFEAY 409
Camk2g_3  QTAMEPQTTVHNAERDGKSTESCNTETTEDKLRQIEKITEQLEIAINGDFEAY 386

Camk2g_1  TKICDPGLTSFEPEALGNLVGMDFHKFYFELLSKSNKPHITTLINPVHVGEDAACI 480
Camk2g_2  TKICDPGLTSFEPEALGNLVGMDFHKFYFELLSKSNKPHITTLINPVHVGEDAACI 469
Camk2g_3  TKICDPGLTSFEPEALGNLVGMDFHKFYFELLSKSNKPHITTLINPVHVGEDAACI 446

Camk2g_1  AYIRLQYDGGGRPRGTSQSEETRVWHRDOKDLRNVYHCSGAPAPLQ 529
Camk2g_2  AYIRLQYDGGGRPRGTSQSEETRVWHRDOKDLRNVYHCSGAPAPLQ 518
Camk2g_3  AYIRLQYDGGGRPRGTSQSEETRVWHRDOKDLRNVYHCSGAPAPLQ 495