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

Heng-Yu Chang, Kyra Minahan, Julie A. Merriman and Keith T. Jones*

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 (metll). 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; Mehmann, 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). Therefore, CA-CamKII plays a necessary and sufficient role in transducing the oscillatory Ca2+ signal into cell cycle resumption, but not into cortical granule release.

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 C57B16 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 MEMα 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 (Cutbertson 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 ATGCCAATCTGCAATGCGGAGCAGCGGCA; Rev GATGCTGACATCGGCGCGGACAGCAGCGGCA; β2, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; γ1, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; δ, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; α2, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; α, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; β, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; γ, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC; δ, Fwd ATGCAGATCGCATACATGCGGCAGGAGCAGGGC.
and Rev GTACGTGACCTGCAGCGGGGC; \( \gamma \) Fwd. ATGCCAATTCCG- GGCATGGCCACCCACCG; and Rev. GTACGTGACCTGCAGCGGGC- TTGGG; 81-3 Fwd ATGCCAATTCCGACCTGGTTCGACCACCC; 81 Rev. GTACGTGACCTGCAGCGGGGATGGTACTGGG; 82 Rev GTACGTG- GACATGTGGTGGCACAAAG; 83 Rev. GTACGTGACCTGCAGCGGGC- TTGATGATGG. Ethidium bromide-stained gel fragments were excised and purified prior to TA ligation into the pGEM T-Easy vector or pRN3 vector for sequencing. CamKII\( \gamma \) cRNA was prepared using a modified pRN3 vector (Reis et al., 2006). cRNA was synthesized as described previously (Chang et al., 2004). CamKII antisense morpholino (CamKII-MO) 5'-ACGTGCTGTCAGATCTACCGTGGC-3' (Gene Tools LLC), and a 5-base-mismatch morpholino (CamKII-5MM-MO) 5'-ACCTCC- GTGCAAGTCACGCGGCC-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 *Lentus culinaris* lectin at 10 \( \mu \)g/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 Ca\(^{2+}\) measurement, oocytes were incubated for 30 minutes with 5 \( \mu \)M 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 CamKII\( \gamma \) (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 \( \mu \)g/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\times/1.2 NA UPLSAPO oil-immersion objective.

**RESULTS AND DISCUSSION**

CA-CamKII induces meiotic resumption in mouse eggs (Knott et al., 2006; Madgwick et al., 2005). CamKII is therefore thought to be the downstream kinase activated by the sperm Ca\(^{2+}\) 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 meII arrest.

**Eggs express only the CamKII\( \gamma \) 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 CamKII\( \gamma \) (Fig. 1), consistent with previous EST screens of mouse eggs (Evskiov 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 (\( \alpha \), \( \beta \), \( \delta \) or whole ovary (\( \gamma \)) as positive controls (Fig. 1).

CamKII\( \gamma \) 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 CamKII\( \gamma \) (see Fig. S2 in the supplementary material). Therefore, we conclude that mouse eggs express predominantly CamKII\( \gamma \), and if other CamKII products are present then they are likely to be other splice variants of CamKII\( \gamma \) and present in much lower abundance than CamKII\( \gamma \).

**CamKII\( \gamma \) knockdown does not inhibit oocyte maturation**

Our strategy was to knockdown CamKII\( \gamma \) expression in mature eggs using an antisense morpholino (MO). We injected CamKII-MO into germline 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 CamKII\( \gamma \) 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-CamKII\( \gamma \) 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).

**CamKII\( \gamma \) 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 Ca\(^{2+}\) mimetic Sr\(^{2+}\), 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).

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 CamKIIy3 cRNA was injected together with CamKII-MO at the GV stage, or CA-CamKII cRNA was injected at the metII stage, and both approaches rescued sensitivity to the Sr2+ mimetic Sr2+ (Fig. 3A). Importantly, these two rescue experiments ruled out the possibility that IVM eggs produced following CamKII-MO injection were actually arrested at metII because of a spindle assembly checkpoint. This is because metII 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
fertilization to induce cell cycle resumption. Blocking Ca²⁺ rises in inseminated eggs blocks all events of cell cycle resumption (Kline and Kline, 1992; Lawrence et al., 1998), and, conversely, inducing Ca²⁺ rises in eggs causes exit from meiosis, most effectively when a sperm-like oscillatory signal is induced (Ducibella et al., 2002; Ozil and Huneau, 2001; Saunders et al., 2002). These data however demonstrate that there are no CamKII-independent mechanisms of cell cycle resumption employed by sperm.

**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 metII 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 (Markoukaki et al., 2003; Markoukaki 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 CamKIIy3 acts by itself to induce cell cycle resumption in mouse eggs at fertilization.

**Acknowledgements**

This work was funded by an Australian Research Council Discovery Project (DP0986217) to K.T.J.

**Supplementary material**

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

**References**


CamKII\textsubscript{3} and egg activation


Lawrence, Y., Ozil, J. P. and Swann, K. (1998). The effects of a Ca\textsuperscript{2+} chelator and heavy-metal-ion chelators upon Ca\textsuperscript{2+} oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca\textsuperscript{2+} increases. Biochem. J. 335, 335-342.


