

Calmodulin-dependent protein kinase gamma 3 (CamKII γ 3) 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 CamKII γ 3 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 CamKII γ 3 or constitutively active CamKII cRNAs. Although CamKII-morpholino-treated eggs fail to exit metaphase II arrest, cortical granule exocytosis is not blocked. Therefore, CamKII γ 3 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 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 (Markoulaki et al., 2003; Markoulaki 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 α with 20% fetal calf serum (Invitrogen) at 37°C in 5% CO₂ (Reis et al., 2006). Milrinone (1 μ M) 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'): α 1, Fwd ATGCGAATTCGCCATGCTGCTCTTCTCACGC and Rev GATCGTCGACATGCGGCAGGACGGA; α 2, Fwd ATGCAGATCTGCCATGGCTACCATCACCTGC and Rev GATCGTCGACATGCGGCAGGACGGA; β , Fwd ATGCGAATTCGCCATGGCCACCACGGT

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and Rev GATCGTCGACCTGCAGCGGGGC; γ , Fwd ATGCGAATTC-GCCATGGCCACCACCGC and Rev GATCGTCGACCTGTAGCG-TTGCGG; $\delta 1$ -3 Fwd ATGCGAATTCGCCATGGCTTCGACCACC; $\delta 1$ Rev GATCGTCGACGTTGATGGGTACTGTGG; $\delta 2$ Rev GATCGTC-GACGATGTTTTGCCACAAAG; $\delta 3$ Rev GATCGTCGACTTGACAA-TTAGTTGATGG. 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 $\gamma 3$ cRNA was prepared using a modified pRN3 vector (Reis et al., 2006). cRNA was synthesized as described previously (Chang et al., 2004). CamKII γ antisense morpholino (CamKII-MO) 5'-ACGTCGGTGCACAGTCACCGCCGCC-3' (Gene Tools LLC), and a 5-base-mismatch morpholino (CamKII-5MM-MO) 5'-ACCTCC-GTGCAGAGTCACGGCGGCC-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 μ 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 μ M fluo4-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 γ (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 μ 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 metII arrest.

Eggs express only the CamKII $\gamma 3$ 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 γ (Fig. 1), consistent with previous EST screens of mouse eggs (Evsikov 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).

CamKII γ 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 $\gamma 3$ (see Fig.

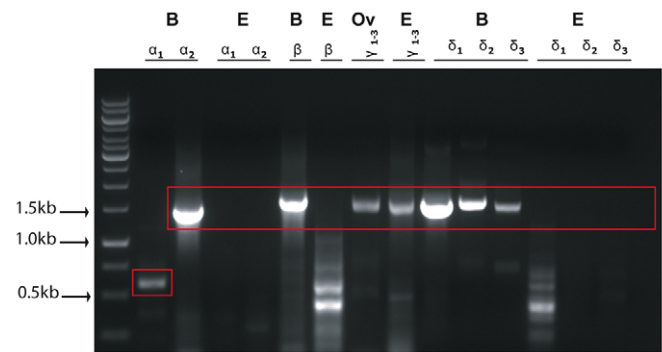


Fig. 1. CamKII $\gamma 3$ is the only isoform of CamKII expressed in mouse eggs. cDNA from brain (B), whole ovary (Ov) or eggs (E) was used to probe for CamKII isoforms as indicated. Isoform-specific primers were used for CamKII $\alpha 1$, $\alpha 2$, β , γ , $\delta 1$, $\delta 2$ and $\delta 3$. A PCR product of the predicted size (boxed) was observed in brain (CamKII $\alpha 1$, $\alpha 2$, β , $\delta 1$, $\delta 2$ and $\delta 3$) or ovary (CamKII γ). Using egg cDNA, only a PCR product for CamKII γ was observed. All prominent bands were sequenced. Only the boxed bands were CamKII γ s. The prominent egg CamKII γ was demonstrated by sequencing to be CamKII $\gamma 3$.

S2 in the supplementary material). Therefore, we conclude that mouse eggs express predominantly CamKII $\gamma 3$, and if other CamKII products are present then they are likely to be other splice variants of CamKII γ and present in much lower abundance than CamKII $\gamma 3$.

CamKII γ knockdown does not inhibit oocyte maturation

Our strategy was to knockdown CamKII $\gamma 3$ expression in mature eggs using an antisense morpholino (MO). We injected CamKII-MO into germinal 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 $\gamma 3$ 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 γ 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 γ 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

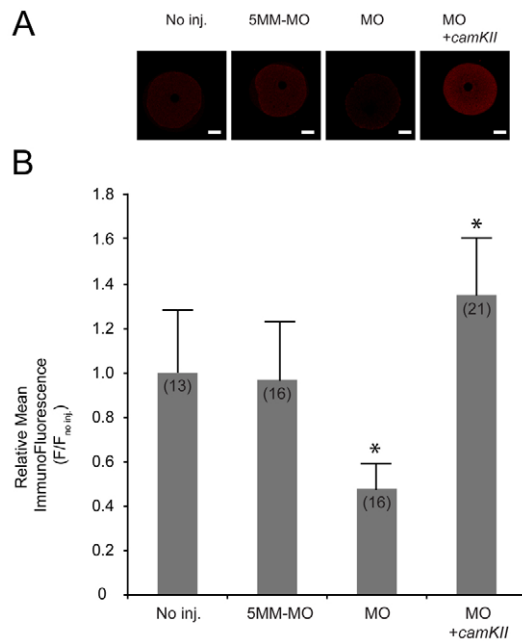


Fig. 2. Efficacy of CamKII MO knockdown. (A) Representative confocal equatorial sections following CamKII γ immunofluorescence in mouse GV oocytes. Twelve hours previously, oocytes had been microinjected with CamKII-MO (MO), 5-base-mismatch CamKII-MO (5MM-MO) or CamKII γ 3 cRNA (*camKII*) as indicated. Oocytes were cultured for 12 hours in milrinone-containing medium. (B) Mean fluorescence levels of oocytes used in A. *y*-axis shows fluorescence measured as the average pixel intensity of the whole oocyte (arbitrary 8-bit scale), relative to the mean intensity of the non-injected oocytes. These data demonstrate a large reduction in immunofluorescence following CamKII-MO, but not 5MM-MO, treatment, as well as the ability of the CamKII γ 3 cRNA to raise CamKII γ 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.

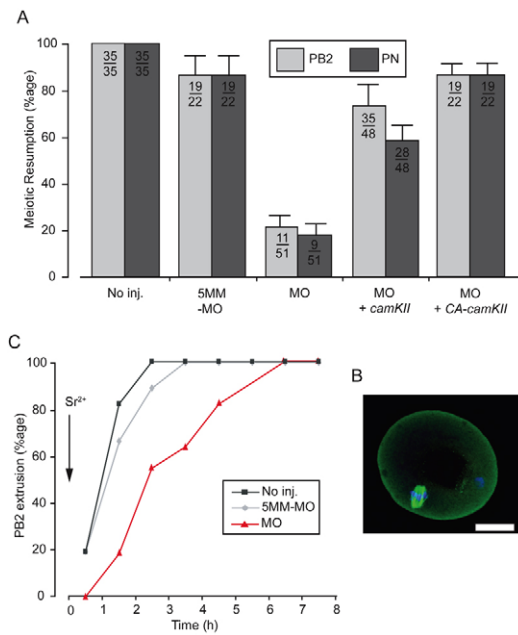


Fig. 3. CamKII MO inhibits exit from metaphase II arrest.

(A) Percentage meiotic resumption from metaphase II arrest, as measured by second polar body (PB2) extrusion and pronucleus (PN) formation in in vitro matured mouse eggs that had been microinjected (or not injected, No inj.) at the GV stage with CamKII-MO (MO), a 5-base-mismatch CamKII-MO (5MM-MO), CamKII γ 3 cRNA (*camKII*), constitutively active CamKII (*CA-camKII*) or buffer. Eggs were parthenogenetically activated using Sr²⁺-containing medium. Within the columns, numerator indicates number of eggs displaying meiotic resumption, and denominator indicates total number of eggs used. Error bars indicate s.d. (B) Equatorial confocal image from a CamKII-MO-injected egg 2 hours after Sr²⁺ activation. Green, tubulin; blue, Hoechst. The egg remains arrested with an intact spindle. Scale bar: 25 μ m. (C) Cumulative PB2 extrusion rate, expressed as a percentage of the total number extruding polar bodies, plotted against the time of extrusion. Egg activation was performed using Sr²⁺-containing medium (added at *t*=0 hours) for eggs used in A as indicated.

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 CamKII γ 3 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 Ca²⁺ 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, cytoskeletal 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

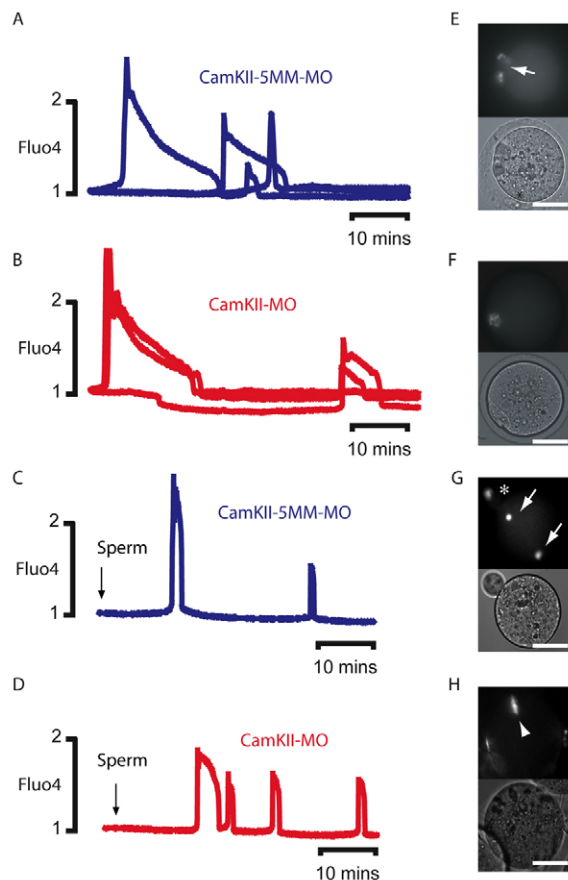


Fig. 4. CamKII MO does not block Ca^{2+} spiking activity. (A-D) Ca^{2+} indicator dye fluorescence changes in mouse eggs following incubation in Sr^{2+} -containing medium (A,B) or sperm addition (C,D). Eggs were injected with 5-base-mismatch CamKII-5MM-MO (A,C) or CamKII-MO (B,D) at the GV stage; Ca^{2+} recordings were started following in vitro maturation. Fluo4 fluorescence at time t minutes is represented as a ratio with respect to fluorescence at $t=0$ minutes (F_t/F_0). Each figure part is representative of at least ten oocytes; A,B show three recordings. (E-H) Hoechst and brightfield images were taken following the recordings in A-D. Sr^{2+} (E,F, at 1 hour) and insemination (G,H, at 3 hours) caused meiotic resumption in eggs injected with CamKII-5MM-MO (E,G) but not CamKII-MO (F,H). (E) Arrow indicates sister chromatids in anaphase. (G) Arrows indicate decondensing female and male chromatin; asterisk, second polar body. (H) Arrowhead indicates a metII spindle. Scale bars: 40 μm .

fertilization to induce cell cycle resumption. Blocking Ca^{2+} rises in inseminated eggs blocks all events of cell cycle resumption (Kline and Kline, 1992; Lawrence et al., 1998), and, conversely, inducing Ca^{2+} 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 do however demonstrate that there are no CamKII-independent mechanisms of cell cycle resumption employed by sperm.

CamKII γ 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 Sr^{2+} addition can be variable (Madgwick et al., 2004). By contrast, a Ca^{2+} 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 Ca^{2+} 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 CamKII γ 3 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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