Development 136, 4077-4081 (2009) doi:10.1242/dev.042143

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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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. Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}-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 Ca^{2+}-induced activation (Markoulaki et al., 2003; Markoulaki et al., 2004).

Despite the importance of CamKII in transducing the Ca^{2+} signal in the cell cycle resumption of eggs, two questions as to its role remain unresolved. Firstly, which CamKII isoform transduces the Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} signal in eggs to similarly induce cell cycle resumption.

School of Biomedical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.

*Author for correspondence (keith.jones@newcastle.edu.au)

Accepted 30 September 2009

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 MEMα with 20% fetal calf serum (Invitrogen) at 37°C in 5% CO_2 (Reis et al., 2006). Milrinone (1 μM) was used to arrest GV oocytes. For parthenogenesis, oocytes were cultured in M2 medium with Ca^{2+} replaced by 3 mM Sr^{2+} (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 ATGCGAATTCGCCATGCTGCTCTTTCTCACGC and Rev GATCGTCGACATGCGGCAGGACGGA; α2, Fwd ATGCAGATCTG-CCATGGCTACCATCACCTGC and Rev GATCGTCGACATGCG-GCAGGACGGA; β, Fwd ATGCGAATTCGCCATGGCCACCACGGT

and Rev GATCGTCGACCTGCAGCGGGGC; γ, Fwd ATGCGAATTC-GCCATGGCCACCACCGC and Rev GATCGTCGACCTGTAGCG-TTGCGG; δ1-3 Fwd ATGCGAATTCGCCATGGCTTCGACCACC; δ1 Rev GATCGTCGACGTTGATGGGTACTGTGG; δ2 Rev GATCGTC-GACGATGTTTTGCCACAAAG; δ3 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. 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′-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 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 Ca^{2+} measurement, oocytes were incubated for 30 minutes with 5 μM 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 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 (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 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 CamKIIγ (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).

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 products are present then they are likely to be other splice variants of CamKIIγ and present in much lower abundance than CamKIIγ3.

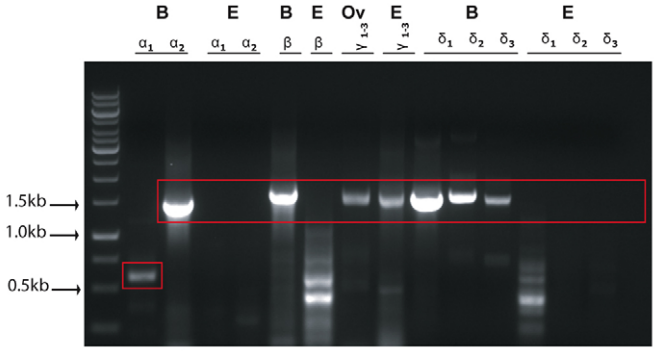


Fig. 1. CamKIIγ3 is the only isoform of CamKII expressed in mouse eggs. cDNA from brain (B), whole ovary (Ov) or eggs (E) was used to probe for CamKII isoforms as indicated. Isoform-specific primers were used for CamKIIα1, α2, β, γ, δ1, δ2 and δ3. A PCR product of the predicted size (boxed) was observed in brain (CamKIIα1, α2, β, δ1, δ2 and δ3) or ovary (CamKIIγ). Using egg cDNA, only a PCR product for CamKIIγ was observed. All prominent bands were sequenced. Only the boxed bands were CamKIIs. The prominent egg CamKIIγ was demonstrated by sequencing to be 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 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 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 Ca^{2+} mimetic Sr^{2+}, which, like sperm, induces an oscillatory activation signal

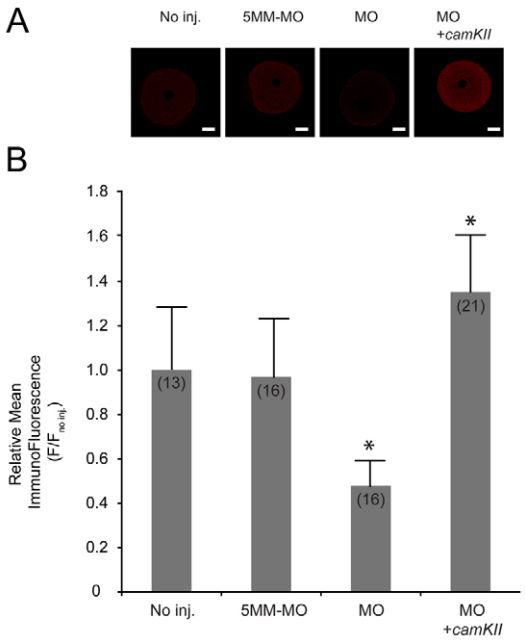


Fig. 2. Efficacy of CamKII MO knockdown. (**A**) Representative confocal equatorial sections following CamKIIγ immunofluorescence in mouse GV oocytes. Twelve hours previously, oocytes had been microinjected with CamKII-MO (MO), 5-base-mismatch CamKII-MO (5MM-MO) or CamKIIγ3 cRNA (*camKII*) as indicated. 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 CamKIIγ cRNA to raise CamKIIγ 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).

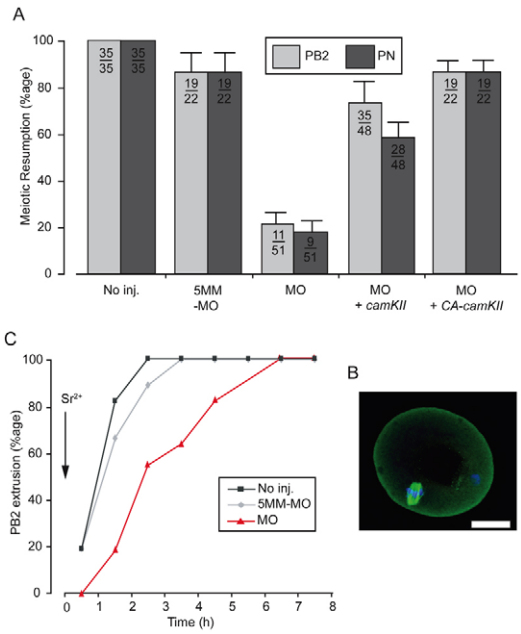


Fig. 3. CamKIIγ MO inhibits exit from metaphase II arrest. (**A**) Percentage meiotic resumption from metII arrest, as measured by second polar body (PB2) extrusion and pronucleus (PN) formation in in vitro matured mouse eggs that had been microinjected (or not injected, No inj.) at the GV stage with CamKII-MO (MO), a 5-base-mismatch CamKII-MO (5MM-MO), CamKIIγ3 cRNA (*camKII*), constitutively active CamKII (*CA-camKII*) or buffer. Eggs were parthenogenetically activated using Sr^{2+}-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 Sr^{2+} activation. Green, tubulin; blue, Hoechst. The egg remains arrested with an intact spindle. Scale bar: 25 μm. (**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 Sr^{2+}-containing medium (added at *t*=0 hours) for eggs used in A as indicated.

(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 Sr^{2+} 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 Sr^{2+} 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 Ca^{2+} signaling by the MO, nor to any essential role of CamKII in Ca^{2+} release, as the Ca^{2+} 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 metII stage, and both approaches rescued sensitivity to the Ca^{2+} mimetic Sr^{2+} (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 Ca^{2+} 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 Ca^{2+} signal, but failed to show signs of meiotic resumption (Fig. 4). These observations are therefore the same as those made with the parthenogenetic agent Sr^{2+}. This would be predicted given that Ca^{2+} is the necessary and sufficient trigger provided by sperm at

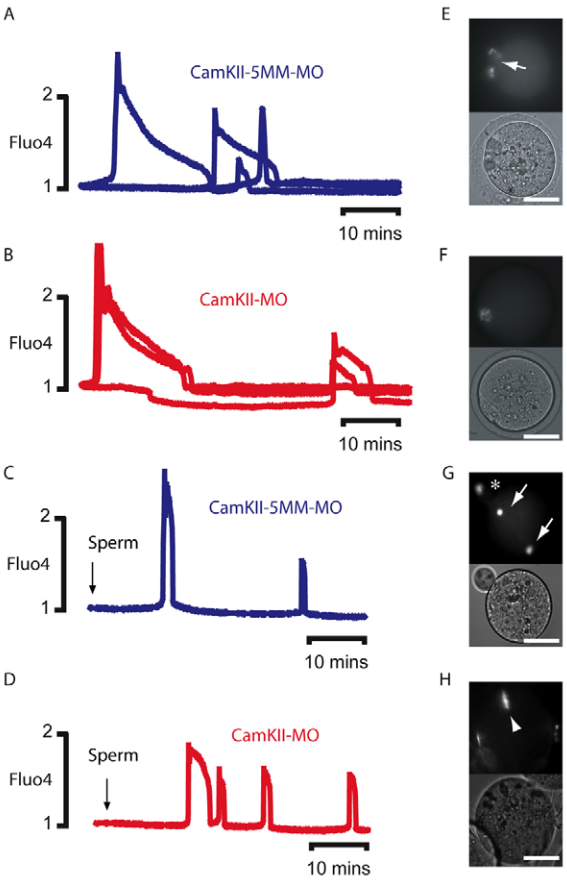


Fig. 4. CamKII MO does not block Ca^{2+} spiking activity. (**A-D**) Ca^{2+} indicator dye fluorescence changes in mouse eggs following incubation in Sr^{2+}-containing medium (A,B) or sperm addition (C,D). Eggs were injected with 5-base-mismatch CamKII-5MM-MO (A,C) or CamKII-MO (B,D) at the GV stage; 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.

CamKIIγ 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 CamKIIγ3 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.

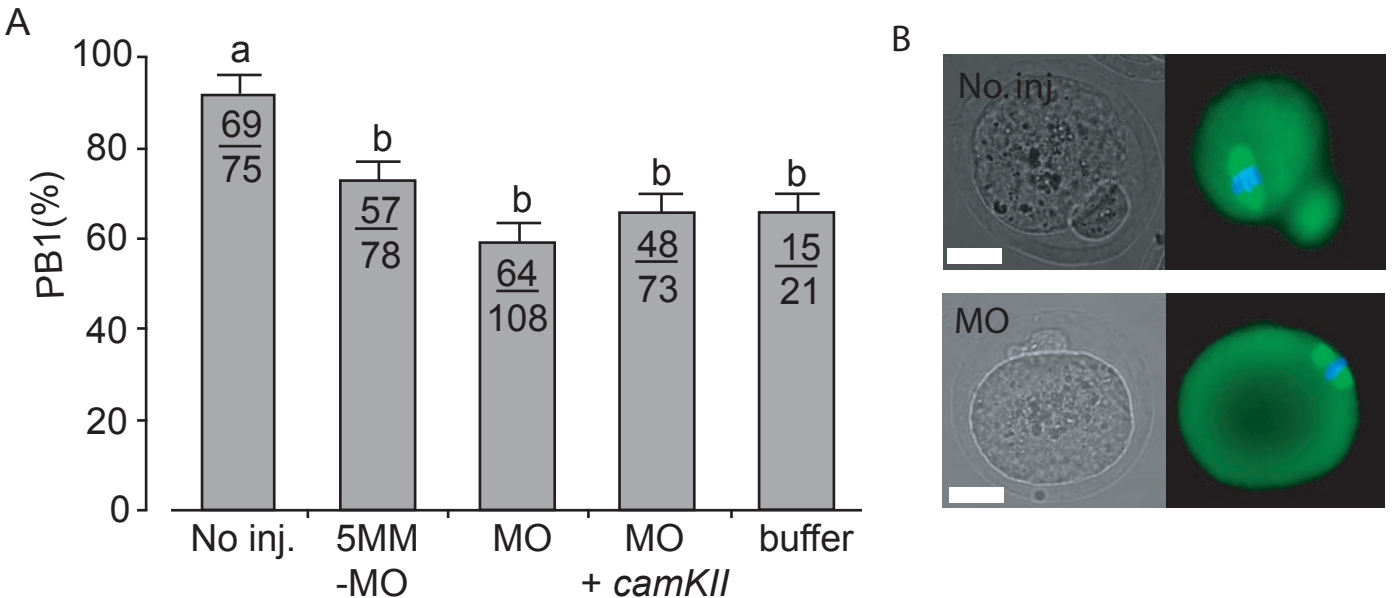
Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/24/4077/DC1

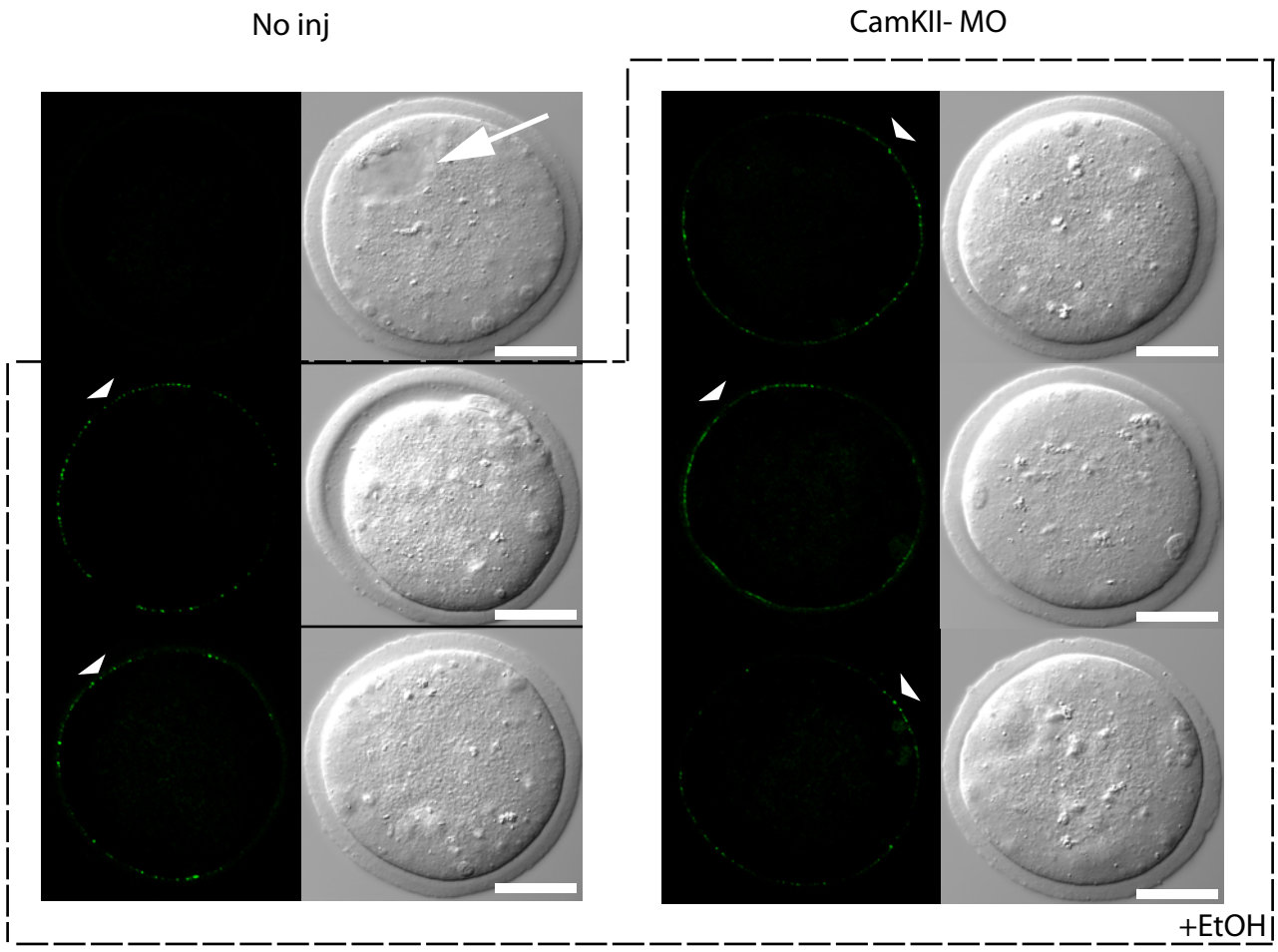
References
Backs, J., Backs, T., Neef, S., Kreusser, M. M., Lehmann, L. H., Patrick, D. M., Grueter, C. E., Qi, X., Richardson, J. A., Hill, J. A. et al. (2009). The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc. Natl. Acad. Sci. USA* **106**, 2342-2347.
Bos-Mikich, A., Whittingham, D. G. and Jones, K. T. (1997). Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* **182**, 172-179.
Chang, H. Y., Levasseur, M. and Jones, K. T. (2004). Degradation of APC^{cdc20} and APC^{cdh1} substrates during the second meiotic division in mouse eggs. *J. Cell Sci.* **117**, 6289-6296.
Cuthbertson, K. S., Whittingham, D. G. and Cobbold, P. H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature* **294**, 754-757.
Ducibella, T. and Fissore, R. (2008). The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev. Biol.* **315**, 257-279.
Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R. M., Kopf, G. S., Fissore, R., Madoux, S. and Ozil, J. P. (2002). Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev. Biol.* **250**, 280-291.
Evsikov, A. V., Graber, J. H., Brockman, J. M., Hampl, A., Holbrook, A. E., Singh, P., Eppig, J. J., Solter, D. and Knowles, B. B. (2006). Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. *Genes Dev.* **20**, 2713-2727.
Hudmon, A. and Schulman, H. (2002a). Neuronal CA^{2+}/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu. Rev. Biochem.* **71**, 473-510.
Hudmon, A. and Schulman, H. (2002b). Structure-function of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. *Biochem. J.* **364**, 593-611.
Jones, K. T. (1998). Protein kinase C action at fertilization: overstated or undervalued? *Rev. Reprod.* **3**, 7-12.
Jones, K. T. (2005). Mammalian egg activation: from Ca^{2+} spiking to cell cycle progression. *Reproduction* **130**, 813-823.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham, D. G. and Kono, T. (1995). Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* **121**, 3259-3266.
Kim, H. and Schuetz, A. W. (1993). Structural and functional differentiation of follicular and oviductal mouse oocytes visualised with FITC-protein conjugates. *Zygote* **1**, 297-307.
Kline, D. and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80-89.
Knott, J. G., Gardner, A. J., Madgwick, S., Jones, K. T., Williams, C. J. and Schultz, R. M. (2006). Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca^{2+} oscillations. *Dev. Biol.* **296**, 388-395.
Lawrence, Y., Ozil, J. P. and Swann, K. (1998). The effects of a Ca^{2+} chelator and heavy-metal-ion chelators upon Ca^{2+} oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca^{2+} increases. *Biochem. J.* **335**, 335-342.
Liu, J. and Maller, J. L. (2005). Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by cytostatic factor. *Curr. Biol.* **15**, 1458-1468.
Lorca, T., Cruzalegui, F. H., Fesquet, D., Cavadore, J. C., Mery, J., Means, A. and Doree, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs. *Nature* **366**, 270-273.
Madgwick, S., Nixon, V. L., Chang, H. Y., Herbert, M., Levasseur, M. and Jones, K. T. (2004). Maintenance of sister chromatid attachment in mouse eggs through maturation-promoting factor activity. *Dev. Biol.* **275**, 68-81.
Madgwick, S., Levasseur, M. and Jones, K. T. (2005). Calmodulin-dependent protein kinase II, and not protein kinase C, is sufficient for triggering cell-cycle resumption in mammalian eggs. *J. Cell Sci.* **118**, 3849-3859.
Madgwick, S., Hansen, D. V., Levasseur, M., Jackson, P. K. and Jones, K. T. (2006). Mouse Emi2 is required to enter meiosis II by reestablishing cyclin B1 during interkinesis. *J. Cell Biol.* **174**, 791-801.
Markoulaki, S., Matson, S., Abbott, A. L. and Ducibella, T. (2003). Oscillatory CaMKII activity in mouse egg activation. *Dev. Biol.* **258**, 464-474.
Markoulaki, S., Matson, S. and Ducibella, T. (2004). Fertilization stimulates long-lasting oscillations of CaMKII activity in mouse eggs. *Dev. Biol.* **272**, 15-25.
Matson, S., Markoulaki, S. and Ducibella, T. (2006). Antagonists of myosin light chain kinase and of myosin II inhibit specific events of egg activation in fertilized mouse eggs. *Biol. Reprod.* **74**, 169-176.
Mehlmann, L. M. (2005). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction* **130**, 791-799.
Morin, N., Abrieu, A., Lorca, T., Martin, F. and Doree, M. (1994). The proteolysis-dependent metaphase to anaphase transition: calcium/calmodulin-dependent protein kinase II mediates onset of anaphase in extracts prepared from unfertilized Xenopus eggs. *EMBO J.* **13**, 4343-4352.
Nixon, V. L., Levasseur, M., McDougall, A. and Jones, K. T. (2002). Ca^{2+} oscillations promote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. *Curr. Biol.* **12**, 746-750.
Ozil, J. P. and Huneau, D. (2001). Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. *Development* **128**, 917-928.
Rauh, N. R., Schmidt, A., Bormann, J., Nigg, E. A. and Mayer, T. U. (2005). Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature* **437**, 1048-1052.
Reis, A., Chang, H. Y., Levasseur, M. and Jones, K. T. (2006). APC^{cdh1} activity in mouse oocytes prevents entry into the first meiotic division. *Nat. Cell Biol.* **8**, 539-540.
Reis, A., Madgwick, S., Chang, H. Y., Nabti, I., Levasseur, M. and Jones, K. T. (2007). Prometaphase APC^{cdh1} activity prevents non-disjunction in mammalian oocytes. *Nat. Cell Biol.* **9**, 1192-1198.
Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K. and Lai, F. A. (2002). PLC zeta: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* **129**, 3533-3544.
Schmidt, A., Duncan, P. I., Rauh, N. R., Sauer, G., Fry, A. M., Nigg, E. A. and Mayer, T. U. (2005). Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Genes Dev.* **19**, 502-513.
Shen, K., Teruel, M. N., Subramanian, K. and Meyer, T. (1998). CaMKIIβ functions as an F-actin targeting module that localizes CaMKIIα/β heterooligomers to dendritic spines. *Neuron* **21**, 593-606.
Shoji, S., Yoshida, N., Amanai, M., Ohgishi, M., Fukui, T., Fujimoto, S., Nakano, Y., Kajikawa, E. and Perry, A. C. (2006). Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20. *EMBO J.* **25**, 834-845.
Swann, K., Saunders, C. M., Rogers, N. T. and Lai, F. A. (2006). PLCzeta(ζ): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Semin. Cell Dev. Biol.* **17**, 264-273.
Tsurumi, C., Hoffmann, S., Geley, S., Graeser, R. and Polanski, Z. (2004). The spindle assembly checkpoint is not essential for CSF arrest of mouse oocytes. *J. Cell Biol.* **167**, 1037-1050.
Walikonis, R. S., Oguni, A., Khorosheva, E. M., Jeng, C. J., Asuncion, F. J. and Kennedy, M. B. (2001). Densin-180 forms a ternary complex with the (α)-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and (α)-actinin. *J. Neurosci.* **21**, 423-433.

```
Camk2g_1        MATTATCTRFTDDYQLFEELGKGAFSVVRRCVKKTSTQEYAAKIINTKKLSARDHQKLER 60
Camk2g_2        MATTATCTRFTDDYQLFEELGKGAFSVVRRCVKKTSTQEYAAKIINTKKLSARDHQKLER 60
Camk2g_3        MATTATCTRFTDDYQLFEELGKGAFSVVRRCVKKTSTQEYAAKIINTKKLSARDHQKLER 60
                ************************************************************

Camk2g_1        EARICRLLKHPNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIHQI 120
Camk2g_2        EARICRLLKHPNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIHQI 120
Camk2g_3        EARICRLLKHPNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIHQI 120
                ************************************************************

Camk2g_1        LESVNHIHQHDIVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGEQQAWFGFAGTPGY 180
Camk2g_2        LESVNHIHQHDIVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGEQQAWFGFAGTPGY 180
Camk2g_3        LESVNHIHQHDIVHRDLKPENLLLASKCKGAAVKLADFGLAIEVQGEQQAWFGFAGTPGY 180
                ************************************************************

Camk2g_1        LSPEVLRKDPYGKPVDIWACGVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDT 240
Camk2g_2        LSPEVLRKDPYGKPVDIWACGVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDT 240
Camk2g_3        LSPEVLRKDPYGKPVDIWACGVILYILLVGYPPFWDEDQHKLYQQIKAGAYDFPSPEWDT 240
                ************************************************************

Camk2g_1        VTPEAKNLINQMLTINPAKRITADQALKHPWVCQRSTVASMMHRQETVECLRKFNARRKL 300
Camk2g_2        VTPEAKNLINQMLTINPAKRITADQALKHPWVCQRSTVASMMHRQETVECLRKFNARRKL 300
Camk2g_3        VTPEAKNLINQMLTINPAKRITADQALKHPWVCQRSTVASMMHRQETVECLRKFNARRKL 300
                ************************************************************

Camk2g_1        KGAILTTMLVSRNFSAAKSLLNKKSDGGVKKRKSSSSVHLMPQSNNKNSLVSPAQEPAPL 360
Camk2g_2        KGAILTTMLVSRNFSAAKSLLNKKSDGGVK-----------PQSNNKNSLVSPAQEPAPL 349
Camk2g_3        KGAILTTMLVSRNFSAAKSLLNKKSDGGVK------------------------------ 330
                ******************************

Camk2g_1        QTAMEPQTTVVHNATDGIKGSTESCNTTTEDEDLKVRKQEIIKITEQLIEAINNGDFEAY 420
Camk2g_2        QTAMEPQTTVVHNATDGIKGSTESCNTTTEDEDLKVRKQEIIKITEQLIEAINNGDFEAY 409
Camk2g_3        ----EPQTTVVHNATDGIKGSTESCNTTTEDEDLKVRKQEIIKITEQLIEAINNGDFEAY 386
                    ************************************************************

Camk2g_1        TKICDPGLTSFEPEALGNLVEGMDFHKFYFENLLSKNSKPIHTTILNPHVHVIGEDAACI 480
Camk2g_2        TKICDPGLTSFEPEALGNLVEGMDFHKFYFENLLSKNSKPIHTTILNPHVHVIGEDAACI 469
Camk2g_3        TKICDPGLTSFEPEALGNLVEGMDFHKFYFENLLSKNSKPIHTTILNPHVHVIGEDAACI 446
                ************************************************************

Camk2g_1        AYIRLTQYIDGQGRPRTSQSEETRVWHRRDGKWLNVHYHCSGAPAAPLQ 529
Camk2g_2        AYIRLTQYIDGQGRPRTSQSEETRVWHRRDGKWLNVHYHCSGAPAAPLQ 518
Camk2g_3        AYIRLTQYIDGQGRPRTSQSEETRVWHRRDGKWLNVHYHCSGAPAAPLQ 495
                **************************************************
```

```
Camk2g_1          CACAAGCTGTATCAGCAGATCAAAGCTGGAGCCTACGATTTCCCATCACCAGAATGGGAC 840
Camk2g_2          CACAAGCTGTATCAGCAGATCAAAGCTGGAGCCTACGATTTCCCATCACCAGAATGGGAC 840
camk2g_3          CACAAGCTGTATCAGCAGATCAAAGCTGGAGCCTACGATTTCCCATCACCAGAATGGGAC 840
Camk2g_cloned     CACAAGCTGTATCAGCAGATCAAAGCTGGAGCCTACGATTTCCCATCACCAGAATGGGAC 840

Camk2g_1          ACAGTCACTCCTGAAGCTAAGAACTTGATCAACCAGATGCTGACCATAAACCCTGCAAAG 900
Camk2g_2          ACAGTCACTCCTGAAGCTAAGAACTTGATCAACCAGATGCTGACCATAAACCCTGCAAAG 900
camk2g_3          ACAGTCACTCCTGAAGCTAAGAACTTGATCAACCAGATGCTGACCATAAACCCTGCAAAG 900
Camk2g_Cloned     ACAGTCACTCCTGAAGCTAAGAACTTGATCAACCAGATGCTGACCATAAACCCTGCAAAG 900

Camk2g_1          CGCATCACGGCCGACCAGGCTCTCAAGCACCCATGGGTCTGTCAACGGTCTACGGTGGCA 960
Camk2g_2          CGCATCACGGCCGACCAGGCTCTCAAGCACCCATGGGTCTGTCAACGGTCTACGGTGGCA 960
camk2g_3          CGCATCACGGCCGACCAGGCTCTCAAGCACCCATGGGTCTGTCAACGGTCTACGGTGGCA 960
Camk2g_Cloned     CGCATCACGGCCGACCAGGCTCTCAAGCACCCATGGGTCTGTCAACGGTCTACGGTGGCA 960

Camk2g_1          TCCATGATGCATCGCCAAGAGACGGTAGAGTGCTTACGCAAATTCAACGCCCGGAGAAAA 1020
Camk2g_2          TCCATGATGCATCGCCAAGAGACGGTAGAGTGCTTACGCAAATTCAACGCCCGGAGAAAA 1020
camk2g_3          TCCATGATGCATCGCCAAGAGACGGTAGAGTGCTTACGCAAATTCAACGCCCGGAGAAAA 1020
Camk2g_Cloned     TCCATGATGCATCGCCAAGAGACGGTAGAGTGCTTACGCAAATTCAACGCCCGGAGAAAA 1020

Camk2g_1          CTGAAGGGTGCCATCCTCACAACCATGCTTGTCTCCAGGAACTTTTCAGCTGCCAAAAGC 1080
Camk2g_2          CTGAAGGGTGCCATCCTCACAACCATGCTTGTCTCCAGGAACTTTTCAGCTGCCAAAAGC 1080
camk2g_3          CTGAAGGGTGCCATCCTCACAACCATGCTTGTCTCCAGGAACTTTTCAGCTGCCAAAAGC 1080
Camk2g_Cloned     CTGAAGGGTGCCATCCTCACAACCATGCTTGTCTCCAGGAACTTTTCAGCTGCCAAAAGC 1080


Camk2g_1          CTATTGAACAAGAAGTCAGATGGCGGTGTCAAGAAAAGGAAGTCGAGTTCCAGCGTGCAC 1140
Camk2g_2          CTATTGAACAAGAAGTCAGATGGCGGTGTCAAG---------------------------- 1113
camk2g_3          CTATTGAACAAGAAGTCAGATGGCGGTGTCAAG---------------------------- 1113
Camk2g_Clone      CTATTGAACAAGAAGTCAGATGGCGGTGTCAAG---------------------------- 1113

Camk2g_1          CTAATGCCACAGAGCAACAACAAAAACAGTCTCGTAAGCCCAGCCCAAGAGCCTGCGCCC 1200
Camk2g_2          ------CCACAGAGCAACAACAAAAACAGTCTCGTAAGCCCAGCCCAAGAGCCTGCGCCC 1167
camk2g_3          ------------------------------------------------------------
Camk2g_Cloned     ------------------------------------------------------------

Camk2g_1          TTGCAGACGGCCATGGAACCACAAACCACCGTGGTACATAATGCTACAGATGGGATCAAG 1260
Camk2g_2          TTGCAGACGGCCATGGAACCACAAACCACCGTGGTACATAATGCTACAGATGGGATCAAG 1227
camk2g_3          ----------------GAACCACAAACCACCGTGGTACATAATGCTACAGATGGGATCAAG 1158
Camk2g_Cloned     ----------------GAACCACAAACCACCGTGGTACATAATGCTACAGATGGGATCAAG 1158

Camk2g_1          GGCTCCACAGAGAGCTGTAACACCACTACAGAAGACGAAGATCTCAAAGTGAGAAAACAA 1320
Camk2g_2          GGCTCCACAGAGAGCTGTAACACCACTACAGAAGACGAAGATCTCAAAGTGAGAAAACAA 1287
camk2g_3          GGCTCCACAGAGAGCTGTAACACCACTACAGAAGACGAAGATCTCAAAGTGAGAAAACAA 1218
Camk2g_Cloned     GGCTCCACAGAGAGCTGTAACACCACTACAGAAGACGAAGATCTCAAAGTGAGAAAACAA 1218

Camk2g_1          GAAATCATTAAGATCACAGAACAACTGATCGAAGCCATCAACAATGGGGACTTTGAGGCC 1380
Camk2g_2          GAAATCATTAAGATCACAGAACAACTGATCGAAGCCATCAACAATGGGGACTTTGAGGCC 1347
camk2g_3          GAAATCATTAAGATCACAGAACAACTGATCGAAGCCATCAACAATGGGGACTTTGAGGCC 1278
Camk2g_Cloned     GAAATCATTAAGATCACAGAACAACTGATCGAAGCCATCAACAATGGGGACTTTGAGGCC 1278

Camk2g_1          TACACGAAGATTTGTGACCCGGGCCTCACATCCTTTGAGCCAGAAGCCCTTGGTAACCTC 1440
Camk2g_2          TACACGAAGATTTGTGACCCGGGCCTCACATCCTTTGAGCCAGAAGCCCTTGGTAACCTC 1407
camk2g_3          TACACGAAGATTTGTGACCCGGGCCTCACATCCTTTGAGCCAGAAGCCCTTGGTAACCTC 1338
Camk2g_Cloned     TACACGAAGATTTGTGACCCGGGCCTCACATCCTTTGAGCCAGAAGCCCTTGGTAACCTC 1338

Camk2g_1          GTGGAAGGAATGGATTTCCATAAGTTTTACTTTGAGAATCTCCTGTCCAAGAACAGCAAG 1500
Camk2g_2          GTGGAAGGAATGGATTTCCATAAGTTTTACTTTGAGAATCTCCTGTCCAAGAACAGCAAG 1467
camk2g_3          GTGGAAGGAATGGATTTCCATAAGTTTTACTTTGAGAATCTCCTGTCCAAGAACAGCAAG 1398
Camk2g_Cloned     GTGGAAGGAATGGATTTCCATAAGTTTTACTTTGAGAATCTCCTGTCCAAGAACAGCAAG 1398

Camk2g_1          CCTATCCACACCACCATCCTAAACCCTCACGTCCACGTGATTGGGGAGGACGCAGCTTGC 1560
Camk2g_2          CCTATCCACACCACCATCCTAAACCCTCACGTCCACGTGATTGGGGAGGACGCAGCTTGC 1527
camk2g_3          CCTATCCACACCACCATCCTAAACCCTCACGTCCACGTGATTGGGGAGGACGCAGCTTGC 1458
Camk2g_Cloned     CCTATCCACACCACCATCCTAAACCCTCACGTCCACGTGATTGGGGAGGACGCAGCTTGC 1458
```


A
100
80
60
40
20
0
PB1(%)
a
b
b
b
b
69
75
57
78
64
108
48
73
15
21
No inj.
5MM
-MO
MO
MO
+ camKII
buffer
B
No. inj.
MO


No inj
CamKII- MO
+EtOH