

# Calmodulin-dependent protein kinase II, and not protein kinase C, is sufficient for triggering cell-cycle resumption in mammalian eggs

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## Summary

Mouse eggs arrest at metaphase II following ovulation and are only triggered to complete meiosis when fertilized. Sperm break the cell-cycle arrest by a long-lasting series of  $Ca^{2+}$  spikes that lead to an activation of the anaphase-promoting complex/cyclosome. The signal transduction pathway is not fully resolved but both protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII) activities increase at fertilization and previous pharmacological studies have implicated both in cell-cycle resumption. We have used a combination of pharmacological inhibitors and constitutively active cRNA constructs of PKC $\alpha$  and CamKII $\alpha$  microinjected into mouse eggs to show that it is CamKII and not PKC that is the sufficient trigger for cell-cycle resumption from metaphase II arrest.

Constitutively active PKC constructs had no effect on the resumption of meiosis but caused an immediate and persistent elevation in intracellular  $Ca^{2+}$  when store-operated  $Ca^{2+}$  entry was stimulated. With respect to resumption of meiosis, the effects of constitutively active CamKII on eggs were the same as sperm. Eggs underwent

second polar body extrusion and pronucleus formation with normal timings; while both securin and cyclin B1 destruction, visualised by coupling to fluorescent protein tags, were complete by the time of polar body extrusion. Induction of a spindle checkpoint by overexpression of Mad2 or by spindle poisons blocked CamKII-induced resumption of meiosis, but the  $Ca^{2+}$  chelator BAPTA did not. Furthermore direct measurement of  $Ca^{2+}$  levels showed that CamKII did not induce exit from metaphase II arrest by raising  $Ca^{2+}$ . Therefore, we conclude that PKCs may play an important role in maintaining  $Ca^{2+}$  spiking at fertilization by promoting store-operated  $Ca^{2+}$  entry, while CamKII transduces cell-cycle resumption, and lies downstream of sperm-induced  $Ca^{2+}$  release but upstream of a spindle checkpoint. These data, combined with the knowledge that CamKII activity increase at fertilization, suggest that mouse eggs undergo cell-cycle resumption through stimulation of CamKII.

Key words: Calcium, Calmodulin-dependent protein kinase II, Fertilization, Mouse, Protein kinase C, Spindle checkpoint

## Introduction

Vertebrate eggs arrest before fertilization at metaphase of the second meiotic division (MetII). Sperm break this arrest by releasing into the egg a sperm-specific phospholipase C-zeta (PLC $\zeta$ ) (Saunders et al., 2002). This induces a rise in intracellular  $Ca^{2+}$  and in so doing the second meiotic division is completed and the newly formed zygote is allowed to progress into the embryonic cell cycles. Destruction of both M-phase (maturation) promoting factor (CDK1/cyclin B1; MPF) activity and the cohesin that holds sister chromatids together is essential in this process of  $Ca^{2+}$ -triggered resumption of meiosis (Hyslop et al., 2004; Madgwick et al., 2004). This dual action on MPF and cohesin is achieved through the destruction of cyclin B1 and securin respectively; a process that is mediated by their polyubiquitination through the E3 ligase activity of the anaphase-promoting complex/cyclosome (APC/C) and subsequent proteolysis by the 26S proteasome complex (Morgan, 1999; Peters, 2002; Zachariae and Nasmyth, 1999).

The  $Ca^{2+}$ -mediated signal transduction pathway at

fertilization is not fully resolved. It is best understood in frog eggs, and here a single sperm-induced  $Ca^{2+}$  rise switches on calmodulin-dependent protein kinase II (CamKII) to activate the APC/C (Lorca et al., 1994; Lorca et al., 1993). The question remains of how well this mechanism of meiosis resumption is evolutionarily conserved. Mammalian eggs show several  $Ca^{2+}$  spikes lasting many hours (Jones, 1998), and not a single 10-minute rise as in frog, however, there is increasing evidence that like frog, fertilization in mammalian eggs is also mediated by CamKII. This is very probable because both frog and mammalian eggs arrest at the same developmental time point and require a  $Ca^{2+}$  signal to break this arrest. However, it has never been tested directly. In support of CamKII being the  $Ca^{2+}$  transducer of mammalian egg meiosis resumption is the observation that increases in CamKII activity are observed during  $Ca^{2+}$ -induced egg activation (Johnson et al., 1998; Markoulaki et al., 2003; Markoulaki et al., 2004; Winston and Maro, 1995) and that CamKII inhibitors are able to block exit from meiosis (Johnson et al., 1998; Tatone et al., 2002). However, interestingly, the same observations have also been

made with respect to protein kinase C (PKC), whose conventional isoforms are activated by  $\text{Ca}^{2+}$  through their C2 domains. Thus PKC activity rises during egg activation when measured directly (Gallicano et al., 1997a), or indirectly by redistribution of various isoforms to the plasma membrane (Eliyahu and Shalgi, 2002; Luria et al., 2000). Furthermore inhibitors of PKC have been reported to prevent mammalian eggs exiting MetII arrest (Gallicano et al., 1997a), and conversely PKC activators are reported to induce completion of meiosis (Colonna et al., 1997; Gallicano et al., 1993; Luria et al., 2000).

It is possible that in mouse eggs a redundant signalling pathway, mediated by either PKC or CamKII ensures exit from MetII arrest. Alternatively, the results achieved using pharmacological inhibitors or activators may represent effects on processes that are upstream of the  $\text{Ca}^{2+}$  signalling pathway, e.g. sperm-egg binding and fusion, or processes that are not normally recruited at fertilization. For example very high concentrations of the PKC activator PMA, a phorbol ester, can induce  $\text{Ca}^{2+}$  spiking in mouse eggs and so cause exit from meiosis (Cuthbertson and Cobbold, 1985). However, this effect is not observed with lower doses that are adequate to induce cell-cycle resumption and so the action of PKC is put downstream and not upstream of the  $\text{Ca}^{2+}$  signal (Gallicano et al., 1993).

The aim of the present study was to determine the respective roles of PKC and CamKII in the meiotic cell-cycle resumption of mouse eggs. We used a combination of pharmacological and molecular approaches to inhibit or induce meiosis exit. We found that CamKII $\alpha$ , but not PKC $\alpha$ , appeared responsible for cell-cycle resumption in mouse eggs and we performed a series of studies to show that CamKII lies downstream of the  $\text{Ca}^{2+}$  signal but upstream of the APC/C. In conclusion we propose that all the cell-cycle events required for full egg activation in mouse are mediated by CamKII.

## Materials and Methods

Chemicals were from Sigma-Aldrich (UK) unless otherwise stated, and of tissue culture or embryo-tested grade where appropriate. KN-92, KN-93, BIM1 and nocodazole were from Calbiochem (UK). Stock solutions were prepared in DMSO (KN-92; BIM1 and nocodazole) or water (KN-93) and stored at  $-20^{\circ}\text{C}$ .

### Gamete collection and culture

Mice were 4- to 8-week-old MFIs (Harlan, UK). Superovulated MetII eggs were collected 12-13 hours after intraperitoneal administration of 5 IU human chorionic gonadotrophin, in mice that had been primed with 7.5 IU pregnant mares' serum gonadotrophin 44-52 hours previously. Cumulus cells were removed by hyaluronidase. Eggs were cultured at  $37^{\circ}\text{C}$  in M2 medium. Parthenogenetic activation of eggs by  $\text{Sr}^{2+}$  was achieved by washing eggs into  $\text{Ca}^{2+}$ -free M2 medium containing 10 mM  $\text{SrCl}_2$  (Madgwick et al., 2004).

For some studies, MetII eggs were loaded with the pharmacological inhibitors ( $<1\%$  stock solutions in M2 medium) for either 10 minutes (nocodazole) or 30 minutes (KN-92, KN93, BIM 1 and BAPTA-AM) before being stimulated to parthenogenetically activate. All inhibitors remained in the medium for the duration of the experiment, except for BAPTA-AM which was washed out. Egg activation rates were assessed by pronucleus formation in formalin-fixed Hoechst 33258 (10  $\mu\text{g}/\text{ml}$ )-stained eggs at 8 hours after addition of a parthenogenetic agent.

### cRNA constructs

The following constructs were used. Constitutively active (CA)-CamKII (a gift from T. Lorca, Montpellier, France), which is a C-terminal deletion of wild-type CamKII $\alpha$  (Lorca et al., 1994; Lorca et al., 1993). Two CA mutants of wild-type PKC $\alpha$  (gifts from P. J. Parker, Cancer Research UK):  $\text{E}^{25}\text{PKC}$ , which carries an alanine to glutamic acid substitution at residue 25, and  $\Delta^{22-28}\text{PKC}$ , in which residues 22-28 are deleted (Genot et al., 1995; Pears et al., 1990). These three CA constructs (CamKII,  $\text{E}^{25}\text{PKC}$ ,  $\Delta^{22-28}\text{PKC}$ ) were cloned into a modified pRN3 vector, designed to produce a cRNA transcript C-terminally coupled to GFP, with maximum stability conferred by the presence of a 5' globin UTR upstream and both 3'UTR and a poly(A)-encoding tract downstream of the gene. Full-length cyclin B1, Mad2, PLC $\zeta$  and securin were cloned as we describe previously (Homer et al., 2005; Hyslop et al., 2004; Madgwick et al., 2004; Nixon et al., 2002).

### Egg microinjection and imaging

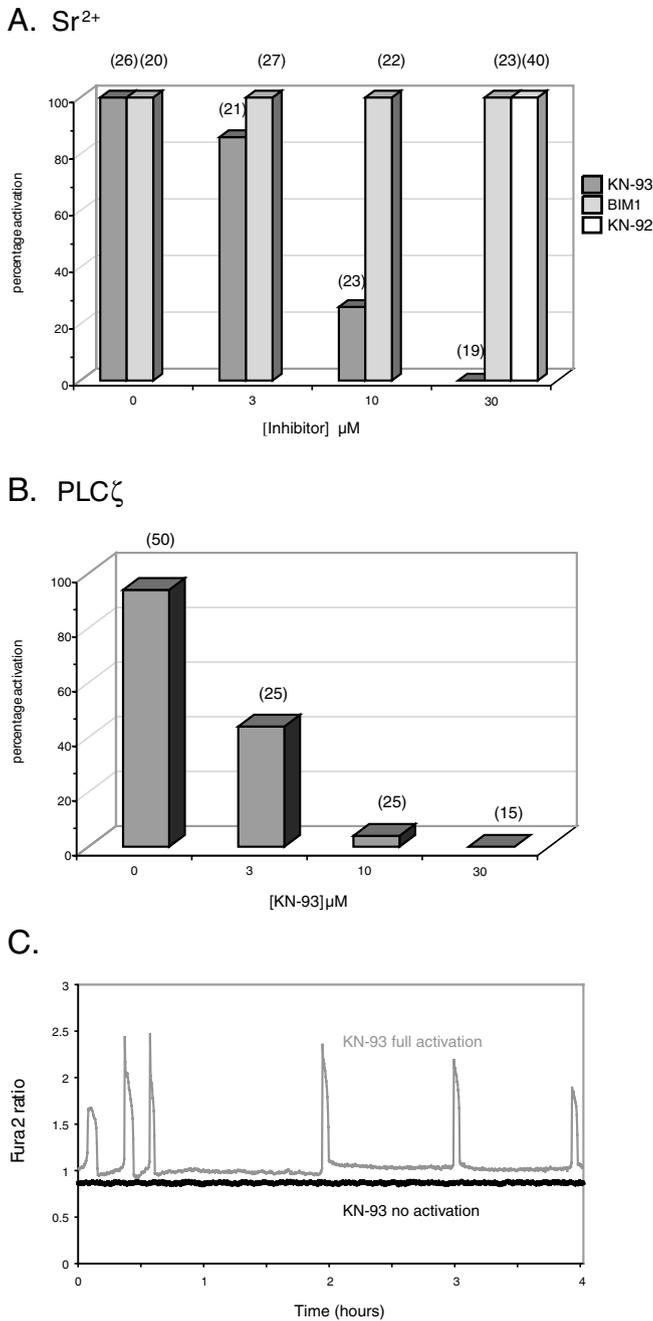
Microinjection of cRNA constructs and of fura2 dextran were made into MetII eggs as described previously (Jones and Nixon, 2000). Briefly eggs were microinjected using fabricated micropipettes and the negative capacitance facility on an electrophysiological amplifier. Such a microinjection procedure ensures high rates of survival ( $>90\%$ ). Egg microinjection and live-cell imaging were performed on the heated stage of an inverted TE300 Nikon microscope fitted for epifluorescence (Nixon et al., 2002). Images from a CCD camera (Micromax 1300Y, Sony) were captured by MetaFluor and MetaMorph imaging software (Universal Imaging Corp, PA, USA) using appropriate filter sets for fura2, CFP, GFP or YFP. These were: for fura2,  $340\pm 12$  nm and  $380\pm 11$  nm excitation filters (Coherent, Leicester, UK) and a DM400 dichroic mirror (Nikon, Kingston upon Thames, UK) fitted with a 435 nm cut-on filter (Chroma, Rockingham, VT); for CFP, a  $430\pm 10$  nm excitation filter (Coherent) and a 51017 BS&M dichroic mirror and emission filter (Chroma); GFP; a  $480\pm 15$  nm excitation filter (Chroma) and a DM505 dichroic mirror fitted with a  $535\pm 40$  nm emission filter (Nikon). Hoechst staining was examined using a 360 nm excitation filter (Chroma) and the DM400 cube used for  $\text{Ca}^{2+}$  imaging.

## Results

### Block in meiotic progression using KN-93 but not BIM1

In mammalian eggs the signal transduction pathway between sperm-induced  $\text{Ca}^{2+}$  spiking and escape from MetII arrest has not been fully resolved. In initial studies, reported here, we used pharmacological inhibitors of either CamKII or PKC to examine if they could block cell-cycle progression in our eggs. BIM1 and KN-93 were used to inhibit PKC and CamKII activity, respectively. Two parthenogenetic stimuli were used,  $\text{Sr}^{2+}$  medium and PLC $\zeta$  because we reasoned that if inhibitors were used in an in vitro fertilization assay they may have spurious effects on sperm binding or fusion.

Pharmacological inhibition of CamKII, but not PKC, blocked exit from meiosis. Eggs treated with  $\text{Sr}^{2+}$  medium failed to resume meiosis with concentrations of the CamKII inhibitor KN-93 greater than 3  $\mu\text{M}$ , but did resume meiosis in the presence of its inactive analogue KN-92 up to a concentration of 30  $\mu\text{M}$  (Fig. 1A). By contrast, inhibition of PKC using BIM1 had no effect on meiotic progression even at the highest dose (30  $\mu\text{M}$ ) tested (Fig. 1A). These are very high doses of BIM1, and although failing to block exit from meiosis, they did induce high rates of cell death ( $\sim 80\%$ ). Cell death was apparent beginning at 30 minutes of incubation and was



**Fig. 1.** Pharmacological inhibition of CamKII, but not PKC, blocked exit from meiosis. (A) To induce completion of meiosis, eggs were incubated in Sr<sup>2+</sup> medium supplemented with doses of KN-93 (CamKII inhibitor), KN-92 (the inactive analogue of KN-93) or BIM1 (PKC inhibitor). Only KN-93 prevented exit from meiosis. (B) Eggs were induced to resume meiosis by PLC $\zeta$  cRNA microinjection, in a range of concentrations of KN-93-supplemented media. The CamKII inhibitor blocked meiotic progression over a similar dose range as that observed with Sr<sup>2+</sup> medium. (C) KN-93 inhibited Ca<sup>2+</sup> spiking. Intracellular Ca<sup>2+</sup> changes were recorded in eggs incubated in Sr<sup>2+</sup> media supplemented with 10  $\mu\text{M}$  KN-93. All eggs that failed to activate ( $n=35$ ) also failed to exhibit Ca<sup>2+</sup> spiking; however, all those eggs that extruded a second polar body and formed pronuclei ( $n=10$ ) had one or more Ca<sup>2+</sup> spikes. Percentage activation rates were assessed at 8 hours by the formation of pronuclei. The number of eggs used is indicated in parenthesis.

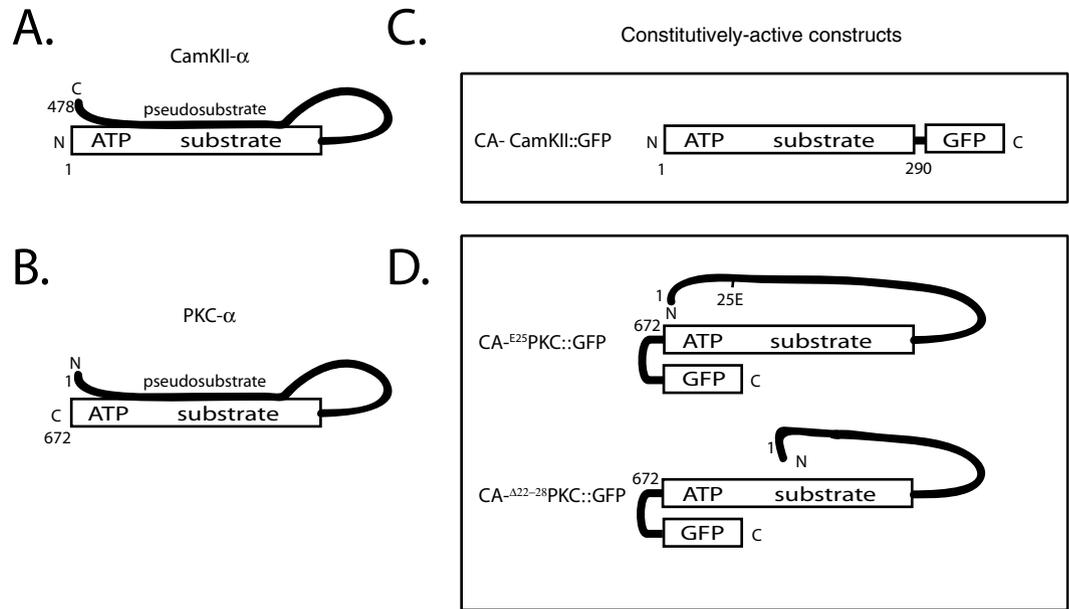
independent of any parthenogenetic stimulation. Other studies have demonstrated that 1–5  $\mu\text{M}$  BIM1 is an effective dose in abolishing PKC activity to around by 96% (Davies et al., 2000; Toullec et al., 1991), therefore the dose range for BIM1 used here is appropriate for it to block PKC activation. When PLC $\zeta$  was used as a second method of parthenogenetic activation, inhibition of CamKII with KN-93 also blocked exit from meiosis, over a similar dose range, suggesting the effects of KN-93 are not specific to Sr<sup>2+</sup> (Fig. 1B).

The data above suggest that PKC activity is not required to break MetII arrest in mouse eggs, but rather point to a role for CamKII in meiosis resumption. However, it is difficult to interpret data based only on inhibition by a pharmacological inhibitor. With respect to the CamKII inhibitor KN-93, it has also been suggested to inhibit Ca<sup>2+</sup> release directly through binding to the calmodulin-binding site of the inositol trisphosphate receptor (InsP<sub>3</sub>R) (Smyth et al., 2002). To examine this further we challenged KN-93-treated eggs, loaded with the Ca<sup>2+</sup> indicator fura2, with Sr<sup>2+</sup> medium ( $n=45$ ; Fig. 1C) or PLC $\zeta$  cRNA ( $n=10$ , not shown). With respect to Sr<sup>2+</sup>, we found no changes in the fura2 ratio in all the 10  $\mu\text{M}$  KN-93-treated eggs that failed to exit MetII arrest ( $n=35$ ; Fig. 1C). However we did observe Ca<sup>2+</sup> spiking in all the eggs that did complete meiosis ( $n=10$ ). In all PLC $\zeta$  cRNA-injected eggs no Ca<sup>2+</sup> spiking was observed in the presence of KN-93 (not shown). Therefore the ability of KN-93 to block egg activation could be attributed to an inhibitory effect on Ca<sup>2+</sup> spiking that may or may not be related to CamKII inhibition.

#### Meiosis resumption through constitutively active CamKII $\alpha$ eggs, but not PKC $\alpha$

We wanted to explore an alternative method to assess the role of PKC and CamKII in the resumption of meiosis. Therefore, we generated cRNA to constitutively active forms of CamKII $\alpha$  and PKC $\alpha$ , coupled to GFP to allow for quantification of expression. Full-length CamKII $\alpha$  (Fig. 2A) was made constitutively active (CA) through the deletion of residues 291–478 (Cruzalegui et al., 1992); PKC $\alpha$  (Fig. 2B) was made constitutively active through the substitution of alanine for glutamic acid at position 25 (E<sup>25</sup>PKC), or the deletion of residues 22–28 ( $\Delta$ <sup>22–28</sup>PKC) (Pears et al., 1990). cRNA to GFP-tagged CA-CamKII; CA-E<sup>25</sup>PKC or CA- $\Delta$ <sup>22–28</sup>PKC (Fig. 2) were microinjected into mouse eggs to assess their ability to induce resumption of meiosis.

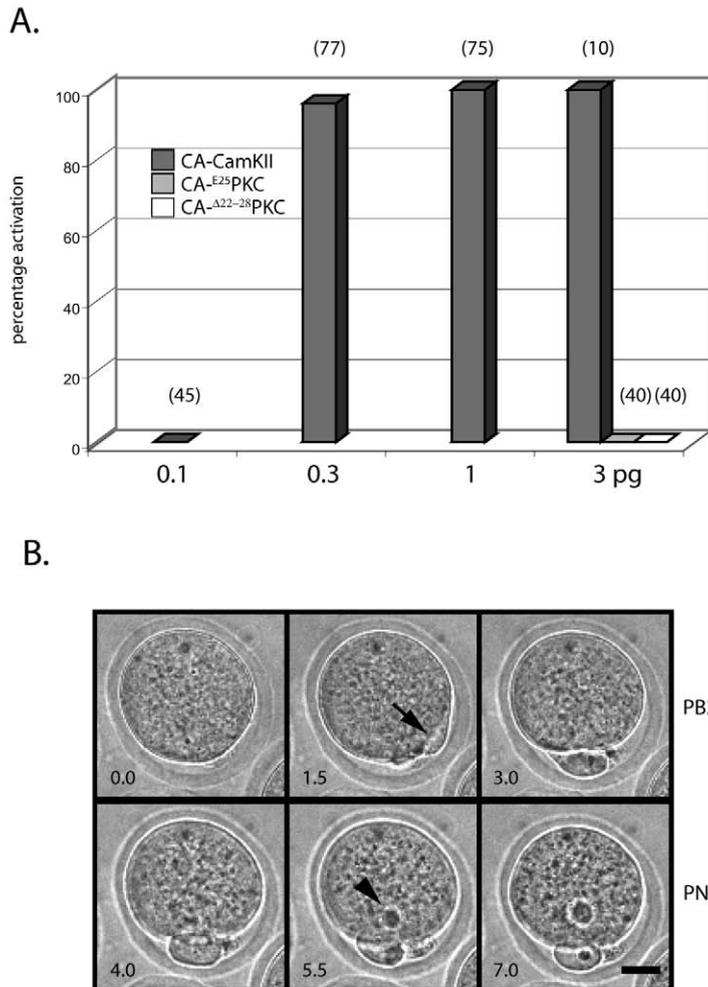
Overexpression of CA-CamKII, but neither of the CA-PKC $\alpha$  constructs, induced the completion of meiosis in mouse eggs, such that 0.3  $\mu\text{g}$  CA-CamKII cRNA resulted in very high rates of pronucleus formation (97%,  $n=77$ , Fig. 3A). At this dose injected eggs appeared non-fluorescent because the amount of CamKII was below the level of detection. We have previously estimated that the limit of our resolution of GFP corresponds to an intracellular concentration of about 50 nM (Madgwick et al., 2004). Therefore, less than 50 nM CamKII was sufficient to induce completion of meiosis. CA-CamKII-injected eggs resumed meiosis with similar timings to those observed with sperm, extruding second polar bodies at about 1–3 hours and forming pronuclei at 4–6 hours (Fig. 3B). By contrast, high doses of both CA-PKC $\alpha$  cRNA constructs (3  $\mu\text{g}$ ) failed to show any of the morphological events of cell-cycle resumption, even after 8 hours of expression. Staining with Hoechst confirmed



**Fig. 2.** Constitutively active CamKII $\alpha$  and PKC $\alpha$  constructs. (A,B) Schematic of wild-type CamKII $\alpha$  (A) and PKC $\alpha$  (B). Constitutively active (CA) mutants of CamKII and PKC were fused C-terminally to GFP. (C) CA-CamKII::GFP; deletion of residues 291-478. (D) CA-E25PKC::GFP; substitution of alanine for glutamic acid at position 25 and CA- $\Delta$ 22-28PKC::GFP; deletion of residues 22-28.

eggs remained arrested at MetII. This was not due to poor expression because GFP levels were readily visible and were calculated to reach low micromolar levels after several hours

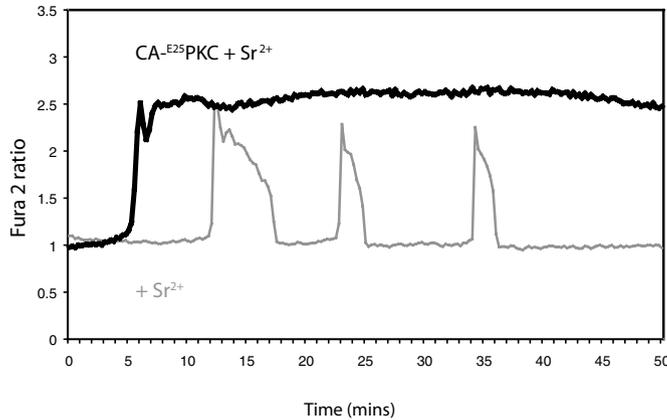
(not shown). A lack of effect is also unlikely to be due to GFP coupling, as previous reports have shown PKC fusions at their C terminus are fully active (Vallentin et al., 2000).



### Effect of CA-PKC on store-operated Ca<sup>2+</sup> entry

Despite previous studies demonstrating activity of C-terminally coupled PKC $\alpha$ ::GFP constructs, and their high expression levels here in mouse eggs, because of their lack of effect on meiosis resumption it was still important to determine if we could observe any physiological effect of their expression in mouse eggs. In a recent elegant study it was shown that full-length PKC overexpression increased the frequency of Ca<sup>2+</sup> spiking in mouse eggs (Halet et al., 2004). The authors concluded that PKC plays a positive role in store-operated Ca<sup>2+</sup> entry (SOCE). We envisaged that the CA-PKC $\alpha$  constructs we have employed here would induce an extreme of this phenomenon if biologically active, and indeed this was the case. Unchallenged eggs that were expressing CA-E25PKC or CA- $\Delta$ 22-28PKC could maintain a steady intracellular Ca<sup>2+</sup> level. However, if Ca<sup>2+</sup> store release was initiated using Sr<sup>2+</sup>, which is known to function by opening the InsP<sub>3</sub>R (Marshall and Taylor, 1994), then eggs were unable to regulate their intracellular Ca<sup>2+</sup> concentration (Fig. 4,  $n=20$ ). Persistent elevation of intracellular Ca<sup>2+</sup> is toxic to cells (Trump and Berezsky, 1992; Trump and

**Fig. 3.** Constitutively active CamKII $\alpha$ , but not PKC $\alpha$ , induces completion of meiosis. (A) CA-CamKII, but not CA-E25PKC or CA- $\Delta$ 22-28PKC, was effective at inducing exit from meiotic arrest. The amount of cRNA injected is plotted against the percentage activation rate, as assessed at 8 hours by the formation of pronuclei. The number of eggs used is indicated in parenthesis. (B) The time course of meiotic progression with CA-CamKII is similar to that observed with sperm, with second polar body extrusion (PB2; indicated by arrow) at about 1.5 hours and pronucleus formation (PN; arrowhead) at about 5.5 hours. Scale bar: 20  $\mu$ m.



**Fig. 4.** The PKC $\alpha$  constructs retain biological activity. Eggs were incubated in Sr<sup>2+</sup>-containing medium 2 hours after microinjection of CA-E<sup>25</sup>PKC cRNA ( $n=20$ ). Eggs that had not been injected with the PKC $\alpha$  construct showed a typical spiking pattern, which led to a completion of meiosis ( $n=20$ ). However, following Sr<sup>2+</sup> addition, eggs expressing CA-E<sup>25</sup>PKC showed an immediate and long-lasting elevation in the fura2 signal, consistent with the reported role of PKCs in SOCE (Halet et al., 2004). We conclude that the PKC $\alpha$  constructs used here retain biological activity.

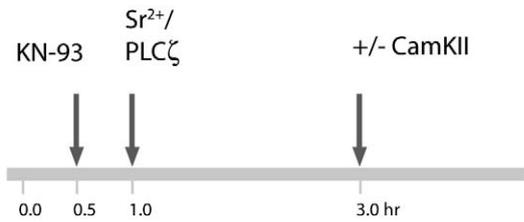
Berezsky, 1995), therefore all eggs treated in this way died. These observations are not as extensive as those of Halet et al. (Halet et al., 2004), but they do suggest that PKC is involved in SOCE. Of more relevance for the present study is our demonstration that CA-PKCs produced physiological responses in eggs, consistent with these CA constructs having biological activity.

#### CamKII activates eggs downstream of Ca<sup>2+</sup>

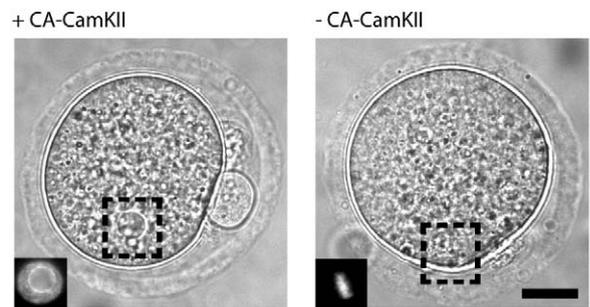
Because pharmacological inhibition of PKC failed to block the resumption of meiosis, and eggs expressing either of the two CA-PKC $\alpha$  constructs remaining arrested at MetII, we concluded that PKC $\alpha$  does not play any direct role in stimulating meiosis resumption. So, we went on to examine the ability of CA-CamKII to induce cell-cycle progression.

We had initially shown the ability of the CamKII inhibitor KN-93 to block meiosis resumption (Fig. 1A,B). Since we observed no Ca<sup>2+</sup> spiking in the presence of KN-93 this could be due to either inhibition of Ca<sup>2+</sup> release independent of CamKII [as suggested by Smyth et al. (Smyth et al., 2002)], or alternatively that CamKII may increase the sensitivity of the egg to Ca<sup>2+</sup> release. We were able to test this directly because we found that the CA-CamKII construct could reverse the effects of KN-93. Parthenogenetic stimulation using either Sr<sup>2+</sup> medium ( $n=56$  eggs) or PLC $\zeta$  ( $n=55$  eggs) induced cell-cycle resumption in only a small number of eggs when 10  $\mu$ M KN-93 was added to the media (Sr<sup>2+</sup>, 4/26; PLC $\zeta$ , 0/25). However, eggs could be induced to complete meiosis if a further 3 pg of CA-CamKII was injected (Sr<sup>2+</sup> 30/30; PLC $\zeta$  30/30; Fig. 5). Using the CA-CamKII construct we found that CamKII is the downstream transducer of the Ca<sup>2+</sup> signal, rather than it acting to sensitise Ca<sup>2+</sup> release. This was shown by three approaches. Firstly, CA-CamKII-induced cell-cycle resumption was not associated with Ca<sup>2+</sup> spiking (Fig. 6A). Secondly, in eggs that

A.



B.

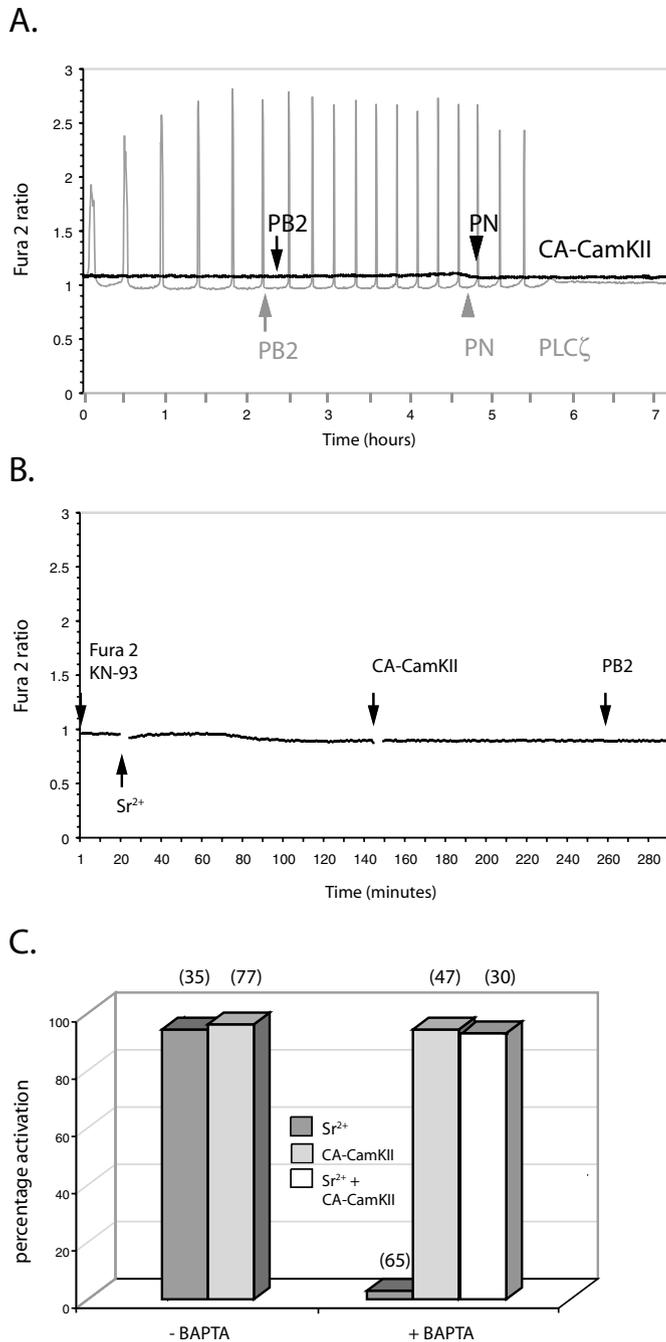


**Fig. 5.** CA-CamKII could overcome the inhibitory effects of KN-93 on eggs. (A) Experimental design. Eggs were treated with a parthenogenetic stimulus (Sr<sup>2+</sup>/PLC $\zeta$ ) but were kept arrested at MetII by 10  $\mu$ M KN-93 for a period of 2 hours. CA-CamKII cRNA was then microinjected into eggs to determine if it could overcome the inhibitory effects of KN-93. (B) Following microinjections of 3 pg CA-CamKII cRNA all eggs activated (30/30 Sr<sup>2+</sup>; 30/30 PLC $\zeta$ ); inset shows decondensed chromatin stained with Hoechst. The vast majority of control eggs not microinjected with CA-CamKII cRNA remained arrested at MetII (rates of resumption of meiosis: 4/26 Sr<sup>2+</sup>; 0/25 PLC $\zeta$ ); inset (right) shows the condensed chromatin of a MetII spindle stained with Hoechst. Scale bar: 20  $\mu$ m.

received a parthenogenetic stimulus but were arrested at MetII with KN-93, CamKII rescued meiosis resumption but not Ca<sup>2+</sup> spiking (Fig. 6B). Thirdly, the Ca<sup>2+</sup> chelator BAPTA blocked meiosis resumption induced by Sr<sup>2+</sup> but not by CamKII; and critically CamKII would also cause resumption of meiosis in eggs treated with Sr<sup>2+</sup> and BAPTA (Fig. 6C). These experiments taken together show that CamKII is the downstream transducer of Ca<sup>2+</sup> in meiosis resumption. CamKII does not induce cell-cycle resumption by any ability to raise intracellular Ca<sup>2+</sup>.

#### CamKII induces cyclin B1 and securin degradation

If CamKII is the downstream effector of Ca<sup>2+</sup> at fertilization it should mimic the ability of sperm to induce degradation of both cyclin B1 and securin. Previously we have shown their degradation begins about 10 minutes after sperm-egg fusion and is complete by second polar body extrusion (Chang et al., 2004; Hyslop et al., 2004; Nixon et al., 2002). Therefore, eggs were microinjected with cRNA to either cyclin B1::YFP or securin::CFP and we monitored their levels of expression following a further microinjection of CA-CamKII. These experiments were made possible by the very low dose of CA-



CamKII used (0.3 pg injection), which although tagged to GFP, remained below the level of detection using our CCD camera. In support of CamKII transducing the Ca<sup>2+</sup> signal at fertilization, we observed degradation of both these constructs with a similar profile to that reported for fertilization. Cyclin B1::YFP (Fig. 7A) and securin::CFP (Fig. 7B) levels dropped to a minimum at the time of second polar body extrusion and remained low until pronuclei formed in the 1-cell embryo. Previously, we had found that these APC/C substrates become stable at this point in the cell cycle (Chang et al., 2004; Nixon et al., 2002), and again this was found here also using CamKII (Fig. 7). Overexpression of both cyclin B1 and securin delayed the polar body extrusion and pronucleus formation relative to

**Fig. 6.** CamKII acts downstream of the fertilization Ca<sup>2+</sup> signal. (A) Microinjection of CA-CamKII induces meiotic progression in the absence of Ca<sup>2+</sup> spiking. Intracellular Ca<sup>2+</sup> was recorded in eggs induced to complete meiosis by microinjection of cRNA to CA-CamKII (black, *n*=12) or PLC $\zeta$  (grey, *n*=8). Second polar body (PB2; arrow) and pronucleus (PN; arrowhead) formation are at the times indicated. (B) Eggs were treated with a parthenogenetic stimulus of Sr<sup>2+</sup> medium but were kept arrested at MetII by KN-93 for a period of 2 hours. CA-CamKII cRNA was then microinjected into eggs. After CA-CamKII injection, eggs exited meiosis but Ca<sup>2+</sup> spiking was not recovered (*n*=15). (C) CA-CamKII-induced meiotic progression was not blocked by the Ca<sup>2+</sup> chelator BAPTA. BAPTA-loaded eggs were either incubated in Sr<sup>2+</sup>-containing medium; or microinjected with CA-CamKII cRNA; or were incubated in Sr<sup>2+</sup>-containing medium followed 2 hours later by CA-CamKII cRNA microinjection. As expected, BAPTA blocked Sr<sup>2+</sup>-induced egg activation but had no inhibitory effect on meiotic progression induced by CA-CamKII, suggesting that the CA-CamKII works downstream of the sperm Ca<sup>2+</sup> signal. Furthermore, in eggs where BAPTA blocked Sr<sup>2+</sup>-induced meiotic progression, microinjection of CamKII cRNA overcame this block. The number of eggs used is indicated in parenthesis.

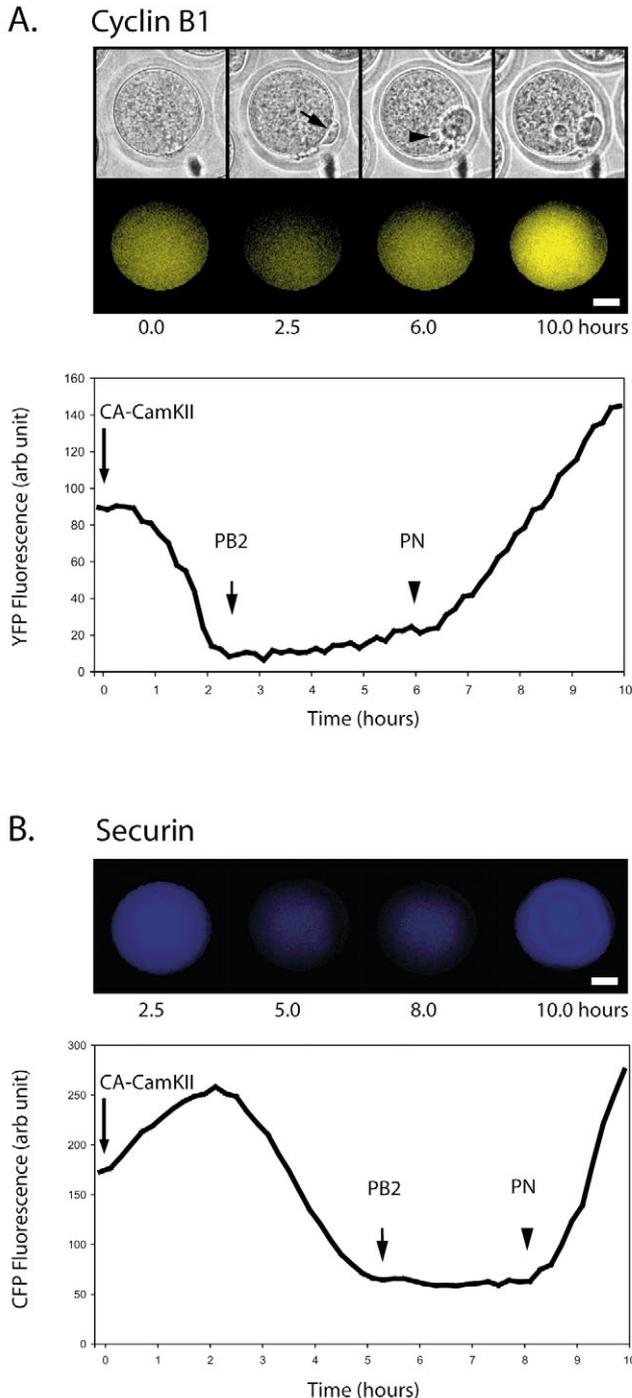
eggs that were not injected with these APC/C substrates. However, importantly, the degradation profiles were the same as we have previously reported for sperm (Chang et al., 2004; Nixon et al., 2002).

#### CamKII activation is blocked by a spindle checkpoint

If CamKII is the immediate transducer of Ca<sup>2+</sup> at fertilization its action should be inhibited by induction of a spindle checkpoint, as is that of sperm. To determine a minimum dose of the spindle poison nocodazole that is necessary to block meiosis progression, eggs were challenged with the parthenogenetic stimulus Sr<sup>2+</sup> and with various doses of nocodazole. We found 100 ng/ml nocodazole to be the minimum effective dose (Fig. 8A), and using this dose there were no gross abnormalities in the alignment of chromatin on the MetII plate (Fig. 8B). Mad2 is an important component of the spindle checkpoint, which inhibits APC/C activation (Fang et al., 1998), and eggs injected with Mad2, coupled with YFP to confirm expression, were unable to exit MetII arrest in the presence of Sr<sup>2+</sup> medium (Fig. 8C). We therefore examined the ability of CA-CamKII to induce meiosis resumption in the presence of either nocodazole or Mad2. When either inducer of the spindle checkpoint was present we found that CamKII was unable to induce meiotic progression and eggs remained arrested at MetII (Fig. 8D). We conclude that CamKII lies immediately downstream of the Ca<sup>2+</sup> signal but upstream of the spindle checkpoint.

#### Discussion

In the present study we found that CamKII, but not PKC, was responsible for Ca<sup>2+</sup>-induced cell-cycle resumption in mouse eggs at MetII. This was shown directly using constitutively active constructs of CamKII and PKC $\alpha$ . CamKII was downstream of the Ca<sup>2+</sup> signal but upstream of the APC/C, such that it was not inhibited by Ca<sup>2+</sup> chelation but was by a spindle checkpoint induced through Mad2 or spindle poisons. Resumption of meiosis induced by CamKII appeared



**Fig. 7.** CA-CamKII promotes the destruction of both cyclin B1 and securin. Eggs were microinjected with cRNA to cyclin B1::YFP or securin::CFP and then with cRNA to CA-CamKII. (A) APC/C activity increased following introduction of CA-CamKII as judged by the degradation of cyclin B1 ( $n=6$ ). Cyclin B1 levels dropped to a minimum at the time of second polar body extrusion and levels remain low until pronuclei formation. Representative brightfield and cyclin B1::YFP images are shown at various times after injection as indicated. (B) A similar degradation profile for securin::CFP is also observed following introduction of CA-CamKII cRNA ( $n=10$ ). (A and B) CA-CamKII cRNA was microinjected at  $t=0$ ; second polar body extrusion (PB2; arrow); pronuclei formation (PN; arrowhead). Scale bar: 20  $\mu\text{m}$ .

indistinguishable from that with sperm. Thus the timings of CamKII-induced exit from meiosis with respect to second polar body extrusion and pronucleus formation were the same as with sperm. By real-time imaging of fluorescent protein constructs we observed both securin and cyclin B1 degradation in MetII eggs following CamKII addition; and more importantly their degradation was complete by second polar body extrusion, as was previously found in fertilized eggs (Nixon et al., 2002). The above data are consistent with a model in which cell-cycle progression from MetII arrest is controlled through CamKII activity.

#### A role for PKC at fertilization, but not in cell-cycle resumption

Through BIM1-mediated PKC inhibition and the use of two CA-PKC $\alpha$  constructs we found no evidence for a role of PKC in the process of cell-cycle progression from MetII arrest. However it has been shown that PKC activity increases at fertilization (Eliyahu and Shalgi, 2002; Gallicano et al., 1997b; Halet et al., 2004; Luria et al., 2000) and so it is likely to play a role in a cell-cycle-independent event. Some PKC inhibitors have been reported to inhibit progression of meiosis (Bement and Capco, 1991; Gallicano et al., 1995; Gallicano et al., 1997a; Gallicano et al., 1993), but as we have found here for the CamKII inhibitor KN-93, inhibitors may affect cell-cycle resumption by inhibiting other proteins or kinases.

Recent experiments point to a role for PKC in Ca<sup>2+</sup> entry via store-operated channels during fertilization (Halet et al., 2004). PKC was found to be involved in the refilling of Ca<sup>2+</sup> stores, by promoting Ca<sup>2+</sup> influx during each Ca<sup>2+</sup> spike. The observation here that the CA-PKCs caused a persistent elevation in the fura2 signal following mobilisation of Ca<sup>2+</sup> stores (by Sr<sup>2+</sup>-induced activation of the inositol trisphosphate receptor) is consistent with the work of Halet et al. (Halet et al., 2004). In their study full length PKC caused the Ca<sup>2+</sup> spiking frequency to increase, however, the fact that the constructs used here were constitutively active obviously led to an extreme effect, and the egg appeared unable to switch off capacitative Ca<sup>2+</sup> entry once it had been initiated by Ca<sup>2+</sup> store release. This makes the CA-PKC constructs valuable tools in studying further the association of PKCs with SOCE, but more relevant to the present findings is that they demonstrate CA-PKCs have biological activity within the egg.

The experiments here and those of Halet et al. (Halet et al., 2004) point to a more indirect effect of PKC on fertilization that may help reconcile some of the inconsistencies in PKC's role at fertilization. It is probable that for some PKC inhibitors a block to cell-cycle progression is caused not because PKC transduces the fertilization Ca<sup>2+</sup> signal for the resumption of meiosis but rather that it is involved in maintaining Ca<sup>2+</sup> spiking through promoting influx.

The CA-PKC constructs used here were both derived from PKC $\alpha$ . There are at least 10 other known members of the PKC superfamily (Mellor and Parker, 1998), so potentially any one of them if present in eggs may have a role at fertilization and specifically in the resumption of meiosis. Blocking Ca<sup>2+</sup> release at fertilization blocks all the cell-cycle-associated events of egg activation (Kline and Kline, 1992; Hyslop et al., 2004) therefore only the Ca<sup>2+</sup>-sensitive members of the PKC superfamily ( $\alpha$ ,  $\beta$  and  $\gamma$ ) can be considered likely candidates

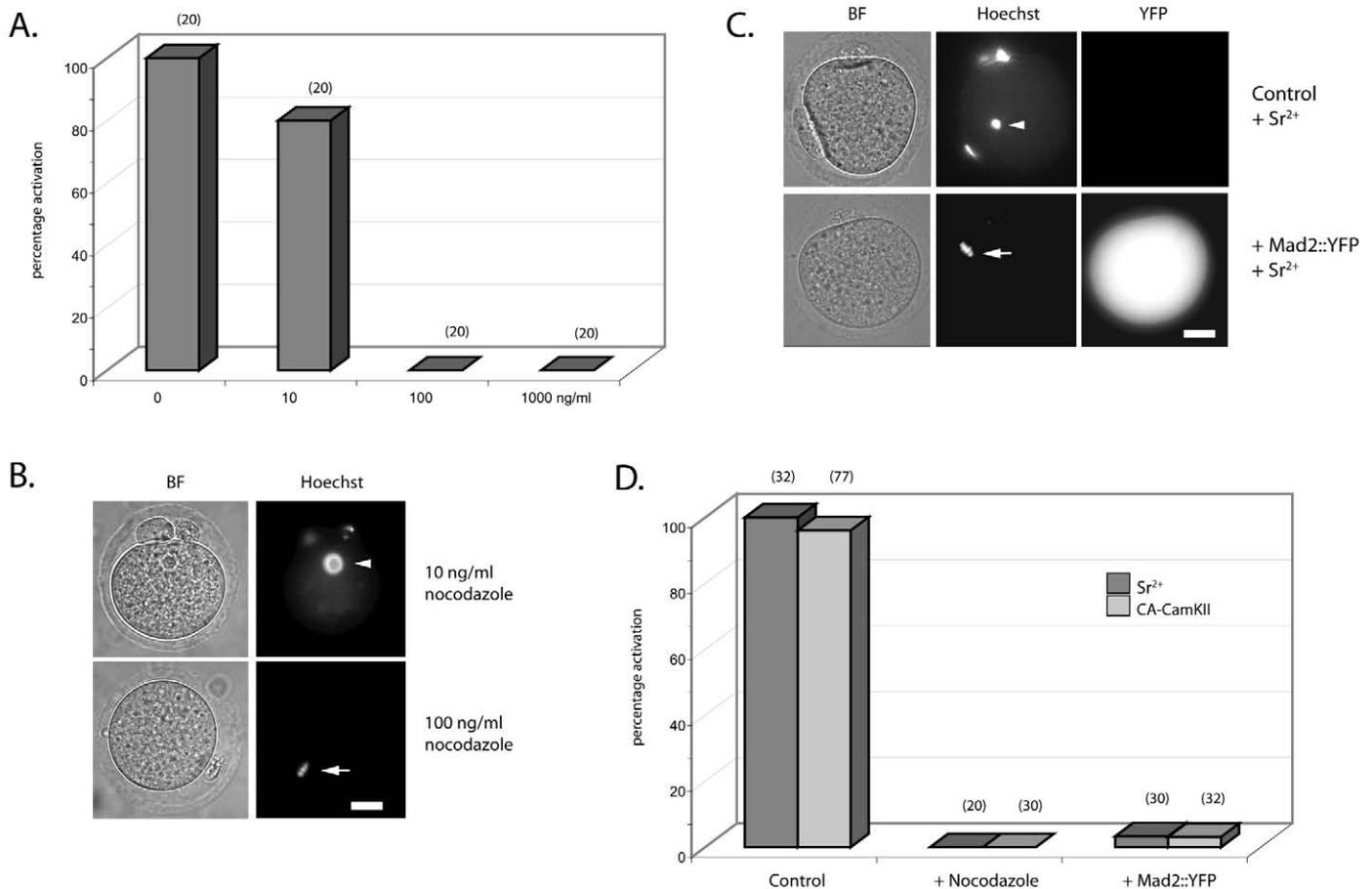
to transduce the  $\text{Ca}^{2+}$  signal at fertilization. While the present data cannot rule out a specific fertilization function of PKC $\beta$  or  $\gamma$ , which cannot be fulfilled by overexpression of PKC $\alpha$ , we feel this unlikely given some of the signalling redundancy of the superfamily; the absence of PKC $\beta$  in some mouse strains (Pauken and Capco, 2000), and the normal fertility of the PKC $\gamma$  knockout mouse (Abeliovich et al., 1993).

### CamKII induces cell-cycle resumption in mouse eggs

The present data show that CA-CamKII $\alpha$  can mimic the cell-cycle resumption normally induced at fertilization. The CamKII construct is made constitutively active by removal of the C-terminal residues 291-478. These residues form part of the so-called autoregulatory and association domains (Hudmon and Schulman, 2002); the CA-CamKII used here comprises mostly of just the catalytic domain. There are several members of the CamKII family ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , and splice variants thereof) that could potentially transduce the  $\text{Ca}^{2+}$  signal for the resumption of meiosis but they all share great homology (89-93%) in their catalytic and autoregulatory domains (Hudmon

and Schulman, 2002). Therefore the finding here that CA-CamKII $\alpha$  can cause meiosis resumption does not necessarily imply that this member of the family is actually involved in the physiological response. This is illustrated nicely by the fact that the present construct is the same as that reported to activate frog eggs (Lorca et al., 1994; Lorca et al., 1993) yet frog does not express CamKII $\alpha$  only CamKII $\beta$ ,  $\gamma$ , and  $\delta$  (Tombes et al., 2003). Therefore it is likely in frog that some other member of the CamKII family transduces the sperm  $\text{Ca}^{2+}$  signal. The fact that the same construct induces cell-cycle resumption in both frog and mouse eggs suggests a conserved mechanism of action that would be consistent with the same meiotic arrest (MetII) in both animals.

In mouse eggs discrete pulses in CamKII activity can be measured in response to the fertilization-associated  $\text{Ca}^{2+}$  spikes (Markoulaki et al., 2004) and on this basis it has been suggested that short bursts of CamKII activity, rather than a steady and continuously elevated level of CamKII, may be needed to drive many of the activation events in mammalian eggs. The present study has achieved apparently normal meiotic progression in eggs exposed to a continuously elevated



**Fig. 8.** CamKII-induced meiotic progression is blocked by a spindle checkpoint. (A-C) Both nocodazole and Mad2 could inhibit Sr<sup>2+</sup>-induced exit from MetII arrest. (A,B) 100 ng/ml nocodazole completely inhibited parthenogenetic activation by Sr<sup>2+</sup> medium as judged by Hoechst staining but 10 ng/ml nocodazole had little effect. (C) Over expression of Mad2::YFP similarly blocked cell-cycle progression. (D) A spindle checkpoint induced by either nocodazole (100 ng/ml) or Mad2 blocked CA-CamKII-induced resumption of meiosis. The number of eggs per treatment group is indicated in parenthesis. In B and C the arrow indicates the MetII spindle and the arrowhead, decondensed chromatin in a pronucleus. Scale bar: 20  $\mu\text{m}$ .

level of CamKII, since polar body extrusion and pronuclear formation occur with similar timings to those observed after fertilization. Although we have not examined the developmental competence of these activated eggs they do undergo a first mitotic division normally (S.M. and K.T.J., unpublished observation) suggesting that they are not severely affected by a non-oscillatory pattern of CamKII activity. However, since we have no current method to modulate CamKII activity in an oscillatory pattern we have no easy comparison to make. It is possible that oscillations in CamKII activity simply reflect the oscillatory  $\text{Ca}^{2+}$  pattern, which has to be so because a sustained high  $\text{Ca}^{2+}$  level is detrimental to cell viability (Trump and Berezsky, 1992; Trump and Berezsky, 1995).

Downstream targets of CamKII are likely to be components of the APC/C

CamKII acts downstream of  $\text{Ca}^{2+}$  but upstream of the spindle checkpoint, which inhibits APC/C activation. The initial study with KN-93, which appeared to stop cell-cycle progression from MetII arrest at least in part by blocking  $\text{Ca}^{2+}$  spiking, had suggested to us that possibly CamKII could work via sensitising the egg to  $\text{Ca}^{2+}$  release; a hypothesis that would be consistent with the known ability of the  $\text{InsP}_3\text{R}$  to bind calmodulin (Taylor, 1998). This ability of KN93 to block  $\text{Ca}^{2+}$  release has also been observed in another study (Smyth et al., 2002). However, using CA-CamKII we find that this ability of KN-93 to block spiking is definitely independent of its ability to block CamKII activity, and so is in agreement with the study of Smyth et al. (Smyth et al., 2002). Thus CA-CamKII could overcome KN-93-mediated egg arrest, but overcoming KN-93-induced arrest was not associated with any re-initiation of  $\text{Ca}^{2+}$  spiking. Also CA-CamKII-mediated meiosis exit was independent of any measurable  $\text{Ca}^{2+}$  rise and was not blocked by the  $\text{Ca}^{2+}$  chelator BAPTA, which has been used in many studies to block exit from meiosis at fertilization (Hyslop et al., 2004; Kline and Kline, 1992; Zernicka-Goetz et al., 1995) and following parthenogenetic stimuli (Sette et al., 1997; Winston et al., 1995). Therefore the results with KN-93 emphasise the need for caution in interpreting inhibitor studies; a fact highlighted by systematic studies of a wide range of available inhibitors (Bain et al., 2003; Davies et al., 2000). The present data show that CamKII acts downstream of the  $\text{Ca}^{2+}$  signal and not via a mechanism that potentiates or initiates  $\text{Ca}^{2+}$  release.

Spindle poisons added to mouse eggs block cell-cycle progression through induction of a spindle checkpoint (Jones et al., 1995; Winston et al., 1995). We found that checkpoint arrest through nocodazole and also overexpression of Mad2, a well known component of the spindle checkpoint (Shah and Cleveland, 2000; Yu, 2002), prevented CamKII-induced exit from meiosis. Mad2 overexpression in other cells similarly induces a spindle checkpoint (Chen et al., 1998; Fang et al., 1998; He et al., 1997). These data would support the suggestion that MetII mouse eggs are not arrested by a spindle checkpoint (Tsurumi et al., 2004), or at least not by Mad2 or spindle checkpoint components that are normally recruited by nocodazole.

The target of CamKII is most probably APC/C<sup>cdc20</sup>. The activity of the 26S proteasome, another potential target for  $\text{Ca}^{2+}$

action, does not increase during mouse egg activation (Hyslop et al., 2004). Induction of a spindle checkpoint would be predicted to prevent the APC/C activator cdc20 (Morgan, 1999; Peters, 2002; Yu, 2002; Zachariae and Nasmyth, 1999) from switching on the APC/C. Good candidates responsible for MetII arrest include Emi1 (Reimann and Jackson, 2002; Reimann et al., 2001) or Erp1/Emi2 (Schmidt et al., 2005; Tung et al., 2005); and both are reported to affect the ability of cdc20 to activate the APC/C. Similarly the c-mos MAP kinase signalling pathway cascade, which has long-been implicated in MetII arrest, activates p90<sup>Rsk</sup> that has been reported to switch on both Bub1 [a spindle checkpoint component (Schwab et al., 2001)] and Emi1 (Paronetto et al., 2004). However, in mouse eggs the sperm does not switch on an inactive APC/C, but instead increases APC/C activity sixfold (Nixon et al., 2002). Also, overexpression of cdc20 or cdc20 mutants that have increased activity do not induce parthenogenetic activation of mouse eggs (Tsurumi et al., 2004). These observations suggest that CamKII may affect the activity of other components of the APC/C. Phosphorylation of the APC/C occurs on several subunits in a complex temporal way (Kraft et al., 2003). Therefore, it remains possible that CamKII can directly phosphorylate the APC/C so as to increase its affinity for its substrates or by increasing its processivity.

The APC/C appears to switch off when pronuclei form in the 1-cell embryo (Fig. 7). This observation probably reflects an inherent inactivation of the APC/C during late G1 period of the cell cycle, rather than a switch off in CamKII activity. APC/C is associated with UbcH10 (Townsend et al., 1997), a ubiquitin-carrier protein, whose function is to supply the APC/C with ubiquitin. When, and only when, the APC/C has fully degraded both securin and cyclin B1 does UbcH10 become a substrate of the APC/C itself (Rape and Kirschner, 2004). Therefore, although not formally tested here, the restabilisation of cyclin B1 and securin at pronucleus formation probably reflects an autonomous inactivation of the APC/C at this time by degradation of UbcH10. However, it may be that the APC/C is under further cell-cycle regulation, for example, at pronucleus formation MAP kinase activity falls (Moos et al., 1995; Moos et al., 1996); therefore it is possible that MAP kinase is responsible for maintaining APC/C activity before this event. Future studies are needed to determine what factors switch off the APC/C at the end of meiosis.

In summary this study has demonstrated that CamKII can induce resumption of meiosis in mouse eggs. They complement the previous observations that CamKII activity increases during mouse fertilization and that a potent peptide CamKII inhibitor blocks resumption of meiosis (Markoulaki et al., 2004). These data, taken together, point to CamKII being the physiological transducer of the  $\text{Ca}^{2+}$  spikes for cell-cycle resumption from MetII arrest. Future studies are now needed to determine if it is CamKII itself, or another kinase substrate of CamKII that is responsible for increasing APC/C activity during fertilization.

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