Possible role for Ca\(^{2+}\) calmodulin-dependent protein kinase II as an effector of the fertilization Ca\(^{2+}\) signal in mouse oocyte activation

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The present study shows that Ca\(^{2+}\) calmodulin-dependent protein kinase II (CaM kinase II) is physiologically activated in fertilized mouse oocytes and is involved in the Ca\(^{2+}\) response pathways that link the fertilization Ca\(^{2+}\) signal to meiosis resumption and cortical granule (CG) exocytosis. After 10 min of insemination, CaM kinase II activity increased transiently, then peaked at 1 h and remained elevated 30 min later when most of the oocytes had completed the emission of the second polar body. In contrast, in ethanol-activated oocytes the early transient activation of CaM kinase II in response to a monotonic Ca\(^{2+}\) rise was not followed by any subsequent increase. Inhibition of CaM kinase II by 20 \(\mu\)mol/l myristoylated-AIP (autocamtide-2-related inhibitory peptide) negatively affected MPF (maturing promoting factor) inactivation, cell cycle resumption and CG exocytosis in both fertilized and ethanol-activated oocytes. These results indicate that the activation of CaM kinase II in mouse oocytes is differently modulated by a monotonic or repetitive Ca\(^{2+}\) rise and that it is essential for triggering regular oocyte activation.

Key words: calcium/cortical granule exocytosis/fertilization/MPF inactivation/protein kinases

Introduction

Mouse oocytes are ovulated, ready to be fertilized, while arrested in metaphase of the second meiotic division (MII). Fertilization triggers a cascade of biochemical events essential for the onset of embryonic development and collectively known as oocyte activation (Yanagimachi, 1994). The primary signalling event observed after sperm–oocyte interaction is an increase in the concentration of oocyte cytosolic Ca\(^{2+}\) followed by sustained oscillations in the intracellular Ca\(^{2+}\) concentration (Cuthbertson and Cobbold, 1985; Miyazaki et al., 1986; Fissore et al., 1992; Sun et al., 1992; Ben-Yosef et al., 1993; Fissore and Robl, 1993; Taylor et al., 1993). This Ca\(^{2+}\) signal is necessary and sufficient for initiating most of the major events of oocyte activation, including a decrease in maturing promoting factor (MPF) activity to overcome cell cycle arrest (Murray et al., 1989; Kline and Kline, 1992; Tombes et al., 1992; Kubiak et al., 1993) and exocytosis of cortical granules (CGs) to prevent polyspermy (Kline and Kline, 1992; Abbott and Ducibella, 2001). It has been suggested that the competence of the oocyte to develop into an embryo is associated with the presence of both a Ca\(^{2+}\) release mechanism to generate a physiological pattern of Ca\(^{2+}\) oscillations and the Ca\(^{2+}\) response machinery to transmit Ca\(^{2+}\) signals to elements involved in controlling the cell cycle and secretion (Carroll et al., 1996; Lawrence et al., 1998; Abbott et al., 1999). At present, even though the mechanisms and dynamics of Ca\(^{2+}\) oscillations at fertilization have been well investigated (Swann and Ozil, 1994; Swann and Lai, 1997; Jones, 1998; Lawrence et al., 1998; Deguchi et al., 2000), the Ca\(^{2+}\) response pathways that link the fertilization Ca\(^{2+}\) signal to resumption of meiosis and CG exocytosis are poorly understood.

Different kinds of evidence suggest that in mammalian oocytes a fundamental component of these pathways is the Ca\(^{2+}\) calmodulin-dependent protein kinase II (CaM kinase II), a multifunctional protein kinase that can selectively control multiple potential targets by localizing to specific regions within the cell (Schulman and Hanson, 1993; Braun and Schulman, 1995). In mouse oocytes, CaM kinase II is present at the metaphase II spindle and in the cortex (Johnson et al., 1998; Hatch and Capco, 2001) and is transiently activated by an artificial monotonic Ca\(^{2+}\) increase such as that induced by ethanol or A23187 (Winston and Maro, 1995; Johnson et al., 1998). Based on the use of peptide or pharmacological inhibitors of CaM kinase II in artificially activated oocytes, it has been proposed that this kinase participates in the regulation of oocyte activation downstream of the Ca\(^{2+}\) signal (Inagaki et al., 1997; Johnson et al., 1998; Tatone et al., 1999). Indeed, the ethanol-induced Ca\(^{2+}\) increase fails to stimulate both meiosis resumption and CG exocytosis in mouse oocytes when CaM kinase II is inhibited by KN-93 (Tatone et al., 1999), a pharmacological antagonist of the enzyme. Moreover, immunofluorescence studies have shown that the active β isoform of CaM kinase II during meiosis resumption localizes to cellular elements participating in this process (Hatch and Capco, 2001).

Although no evidence has proven its involvement in mammalian fertilization, CaM kinase II is a good candidate as a physiological effector of the fertilization Ca\(^{2+}\) signal due to its ability to respond sensitively to a temporal pattern of Ca\(^{2+}\) oscillations (Braun and Schulman, 1995; De Koninck and Schulman, 1998) and to regulate both the cell cycle (Baitinger et al., 1990; Ohta et al., 1990; Lorca et al., 1993; Tombes et al., 1995; Morris et al., 1998) and secretion...
CaM kinase II is activated in mouse oocytes at fertilization

Parthenogenetic oocyte activation
To achieve parthenogenetic activation, oocytes were collected at 17 h post-HCG and exposed to 7% ethanol in M16 medium at 37°C, 5% CO2 for 6.5 min. Treated oocytes were thoroughly washed in M16 medium before processing for the evaluation of the designated parameters.

Oocyte treatment with CaM kinase II inhibitor
For studying the physiological role of CaM kinase II, we selected AIP as a potent and specific inhibitor of the enzyme as previously described (Ishida et al., 1995). In particular, we used myr-AIP to raise the permeability of the inhibitor through the cell membrane.

Oocytes were incubated in M16 medium containing 10 or 20 µmol/l myr-AIP for 60 min at 37°C, 5% CO2 washed in medium and then inseminated or exposed to ethanol.

CaM kinase II assay
CaM kinase II activity was measured according to Johnson et al. (Johnson et al., 1997). Oocytes were washed in PBS, transferred to the assay buffer (115 mmol/l KCl, 5 mmol/l 5,5'-diBrBAPTA, 2.1 mmol/l CaCl2, 5.75 mmol/l MgCl2·6H2O, 2.2 µmol/l PKL, 75 µmol/l genestein, 200 µmol/l PKCψ, 240 mmol/l β-glycerophosphate, 120 mmol/l para-nitrophenyl phosphate, 1 mg/ml of each protease inhibitor aprotinin, trypsin/chymotrypsin inhibitors, chymostatin, leupeptin, pepstatin and 23 mmol/l HEPES, pH 6.8), frozen in N2 and stored at −80°C until use. After thawing, the reaction was started by adding 10 µg autocamtide-2 in each sample as a specific substrate for CaM kinase II and 0.25 µCi [γ-32P]/ml (Amersham Pharmacia Biotech, Italy). To inhibit CaM kinase II, non-membrane permeant AIP was added to the assay buffer at a final concentration of 10 µmol/l. After 30 min at 37°C, assays were stopped by adding tricine sample buffer (BioRad Laboratories, Hercules, CA, USA) and samples were electrophoresed on precast Tris-Tricine 16.5% polyacrylamide gel (BioRad) according to a previously described method (Schagger and von Jagow, 1987) using two different running buffers: the anodal buffer containing 0.2 mol/l Tris pH 8.9 and the cathodal buffer containing 0.1 mol/l Tris pH 8.25, 0.1 mol/l tricine, 1% sodium dodecyl sulphate. Following electrophoresis the gel was fixed for 60 min in 0.2% glacial acetic acid and 0.2 mol/l sodium acetate in 30% ethanol, dried and exposed to a phosphor screen for 12 h at −80°C.

Enzyme activity was semi-quantitatively densitometrically using a BioRad GS-670 computerized imaging densitometer and Molecular Analyst software. The intensities of bands were quantified after background subtractions. For each autoradiogram, 2–3 replicates were performed with 10 oocytes per time point per replicate and ratios of mean band density in the experimental groups to that of control MII arrested oocytes were evaluated. CaM kinase II activity at different time points was expressed as mean ± SEM of ratios obtained from 4–8 autoradiograms.

In the experiments that aimed to establish the effect of myr-AIP on CaM kinase II activation by sperm or by ethanol, the percentage inhibition of kinase activity was calculated by comparing the level of CaM kinase II activity in myr-AIP-treated oocytes with that of untreated oocytes at the equivalent time point, according to the following equation: % inhibition = 1−(CaM kinase II activity of treated oocytes/CaM kinase II activity of untreated oocytes)/100.

Inhibition of CaM kinase II activity under different experimental conditions was expressed as mean ± SEM of ratios obtained from at least three experiments.

Histone H1 kinase assay
MPF activity in mouse oocytes was evaluated by assessing the protein kinase activity of p34-cdc2 cyclin B towards histone H1 according to a previously published method (Gallicano et al., 1997). The autoradiograms were scanned and analysed as described above. After quantification, histone H1 activity in the different experimental groups was expressed as a fraction of activity in metaphase II arrested oocytes. For each experiment, three replicates were performed with 10 oocytes per time point per replicate.

Staining and quantification of cortical granules
Oocytes were fixed in 3.7% (w/v) paraformaldehyde and, after permeabilization with Triton X-100, were incubated with LCA coupled to biotin and then with Texas Red-streptavidin, according to a previously described method (Ducibella et al., 1988). The oocytes were then mounted on slides in 50% (v/v) glycerol.
and CGs in the cortex were visualized by a fluorescence microscope equipped with a 100× objective. The CG density for each oocyte was computed by image analysis based on the same principles as manual counting described previously (Ducibella et al., 1988; Tatone et al., 1999). The images of flat optical fields of cortex resulting from partial compression of the oocyte by the coverslip were captured by a Vario Cam monochrome CCD and then transferred to a PC with image analysis software (KS300, Kontron Elektronik Gmbh, Germany). The density of CGs per 100 μm² for each oocyte was computed by image analysis as the mean of the counts from three equal areas of cortex containing cortical granules. For each group, the percentage loss of CGs from the cortex was calculated by comparing the mean density of CGs of the treated group with the mean density of CGs of the untreated control group (Ducibella and Buetow, 1994), according to the following equation: %CG loss = 1 – (density of CGs in treated group/density of CGs in untreated group)×100.

Statistical analysis
Each group of experiments was repeated at least three times. Data are presented as mean ± SEM. Multiple comparisons of values of CaM kinase II activity were analyzed using Student–Newman–Keuls’s test (SigmaStat for Windows; Jandel Scientific Software Corporation, San Rafael, CA, USA). Comparison between control and myr-AIP treatments was conducted using Student’s t-test. Differences of P < 0.05 were considered significant.

Results
CaM kinase II is activated in fertilized mouse oocytes with a temporal pattern different from that of ethanol-activated oocytes
The aim of this part of the study was to determine whether CaM kinase II is physiologically activated by the sperm-induced Ca²⁺ signal in mouse oocytes.

As Ca²⁺ oscillations start a few minutes after sperm–oocyte fusion (Lawrence et al., 1997), we selected an IVF system that ensured a high degree of synchronization of sperm fusion. By using the Hoechst 33342 dye transfer technique we found that after 10 min of insemination of ZP-free oocytes with acrosome-reacted sperm, 97.5 ± 0.5% of oocytes (polyspermy rate, 60 ± 9%; number fused sperm/oocyte, 1.7 ± 0.15; total oocytes examined = 184) showed at least one fused sperm (data not shown). We monitored CaM kinase II activity at increasing time intervals up to 90 min, when ~90% of oocytes had completed polar body formation. As shown in Figure 1, fertilization resulted in an early increase of kinase activity that was observed after 10 min of insemination. The level of activity then fell progressively until 30 min, increased again after 60 min of insemination and was still elevated 30 min later when most of the oocytes had completed the emission of the second polar body. To investigate whether this temporal pattern of activity was related to the oscillatory pattern of the fertilization Ca²⁺ signal, we determined the profile of CaM kinase II activity in oocytes artificially activated through a monotonic Ca²⁺ rise such as that induced by ethanol. To this end, we extended the analysis of the kinase activity in ethanol-treated oocytes beyond the time period previously investigated by Winston and Maro (Winston and Maro, 1995). As shown in Figure 2, CaM kinase II activity increased transiently 6.5 min after ethanol exposure, consistent with the results of Winston and Maro, but did not display any subsequent increase at 90 min from the beginning of treatment.

Myr-AIP differently affects CaM kinase II activity in fertilized and ethanol-activated oocytes
In this section, we determined whether the stimulation of CaM kinase II by sperm or ethanol was prevented by myr-AIP (Ishida et al., 1995). Previous work has shown that CaM kinase II activation induced by A23187 is greatly inhibited in mouse oocytes when exposed to myr-AIP at a concentration of 10 μmol/l (Johnson et al., 1998). In contrast, our results on fertilized oocytes (Figure 3A) revealed that pretreatment with 10 μmol/l myr-AIP did not produce any detectable effect on CaM kinase II activation induced by sperm. However, when myr-AIP was used at 20 μmol/l an inhibition percentage of ~60% could be achieved at any time point investigated. Myr-AIP concentrations >20 μmol/l were not tested in this study given the possibility that they could interfere greatly with protein kinase C activity (Ishida et al., 1995). The inhibitory effects of

![Figure 1. CaM kinase II activity in fertilized mouse oocytes. (A) Representative autoradiogram showing typical results. (B) Histogram showing CaM kinase II activity at different times after the beginning of insemination. The CaM kinase II inhibitor AIP added to the reaction mixture as a control (10 μmol/l final concentration) greatly suppressed the level of substrate phosphorylation. For each autoradiogram, 2–3 replicates were performed with 10 oocytes per time point per replicate. Data were expressed as mean ± SEM of ratios of band intensity in the experimental groups to that of untreated metaphase II arrested oocytes. Different superscripts indicate statistical differences at P < 0.05.](image)

![Figure 2. CaM kinase II activity in mouse oocytes activated by ethanol. The histogram shows CaM kinase II activity at different times after the beginning of treatment. The CaM kinase II inhibitor AIP added to the reaction mixture as a control greatly suppressed the level of substrate phosphorylation. Data were expressed as mean ± SEM of ratios of band intensity in the experimental groups to that of untreated metaphase II arrested oocytes. Different superscripts indicate statistical differences at P < 0.05.](image)
Myr-AIP observed in our experiments were not due to a failure of sperm-oocyte fusion caused by damage to the oolemma. As assessed using the dye transfer technique, after 10 min of insemination similar numbers of sperm/oocyte were found fused in both the control (1.4 ± 0.3; n = 43 oocytes examined) and the group treated with 20 µmol/l myr-AIP (1.7 ± 0.5; n = 56 oocytes examined; data not shown).

As shown in Figure 3B, in ethanol-treated oocytes an inhibition percentage of ~80% was found when oocytes were pretreated with 10 µmol/l myr-AIP, a concentration ineffective in fertilized oocytes. This value of inhibition was significantly higher than that observed at fertilization in the group treated with 10 µmol/l myr-AIP. A further increase of the antagonist to 20 µmol/l did not result in an increase of the inhibitory effect.

**Effect of myr-AIP on early events of oocyte activation induced by sperm or ethanol**

Given previous evidence for a role of CaM kinase II in the molecular mechanisms underlying exit from M-phase and CG exocytosis (Abbott and Ducibella, 2001; Capco, 2001), we determined whether these events were prevented when CaM kinase II activity was inhibited by myr-AIP in mouse oocytes.

**Effect of myr-AIP on MPF inactivation and meiosis resumption**

In our experiments, mouse oocytes preincubated with 20 µmol/l myr-AIP for 60 min were inseminated for 30 min or treated with ethanol and processed for H1 kinase assay after 30, 60 and 90 min from the beginning of the activating treatments. Results revealed that, when assayed at 30 min post-insemination, histone H1 kinase activity was severely reduced in control oocytes, but remained elevated in oocytes treated with myr-AIP (Figure 4A). However this inhibitory effect was transient, as oocytes treated with myr-AIP and assayed after 60 min from the beginning of insemination revealed a decrease of histone H1 kinase activity to a level not significantly different from that observed in the control group. Similar to that observed in inseminated oocytes, MPF inactivation occurring 30 min after ethanol treatment was prevented when CaM kinase II was inhibited by myr-AIP (Figure 4B) but, in contrast to fertilized oocytes, this effect was still observed at 60 and 90 min post-activation.

Consistent with the effects of myr-AIP on MPF inactivation were those effects on release from metaphase arrest. As shown in Figure 5, 30 min after the beginning of activating treatments only a reduced percentage of inseminated oocytes (Figure 5A) or ethanol-treated oocytes (Figure 5B) had undergone meiosis resumption when pre-loaded with 20 µmol/l myr-AIP. However, when examined later, myr-AIP-treated oocytes subjected to insemination had progressed beyond metaphase, reaching the telophase stage with a rate not significantly different from that of the control group. In contrast, myr-AIP-treated oocytes exposed to ethanol were stably arrested at metaphase.

**Effect of myr-AIP on CG exocytosis**

In this set of experiments mouse oocytes preincubated with 20 µmol/l myr-AIP for 60 min were inseminated or treated with ethanol and, at 60 and 90 min after the beginning of activating treatments, values of CG loss were calculated after CG counting in flat fields of cortex such as those in Figure 6A. Results revealed that CG loss was significantly reduced in inseminated or ethanol-activated oocytes pretreated with myr-AIP in comparison with their relative control groups (Figure 6B). We also observed that after myr-AIP treatment, the value of CG loss in fertilized oocytes was ~2-fold higher than that of ethanol-activated oocytes (not significant).

**Discussion**

In this study our working hypothesis was that CaM kinase II in mouse oocytes may act as an effector of the fertilization Ca\(^{2+}\) signal due to its ability to respond sensitively to Ca\(^{2+}\) oscillations and to regulate both the cell cycle and secretion. The results presented here demonstrate, for the first time, that CaM kinase II is physiologically activated in mouse oocytes in response to fertilizing sperm. At 10 min of insemination, once sperm fusion had occurred, CaM kinase II activity increased and then progressively fell within 30 min of insemination. It peaked at 60 min and remained elevated 30 min thereafter, when most of the fertilized oocytes had extruded the second polar body. Thus, as with parthenogenetic activation (Winston and Maro, 1995; Johnson et al., 1998), even at fertilization CaM

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**Figure 3.** Effect of myr-AIP on CaM Kinase II activation induced by sperm (A) or ethanol (B). CaM Kinase II activity was monitored in oocytes exposed for 60 min to 10 or 20 µmol/l myr-AIP prior to insemination or ethanol treatment. Values represent the mean ± SEM of inhibition percentages calculated as described in Materials and methods. Different superscripts indicate statistical differences at $P < 0.05$.

**Figure 4.** Effect of myr-AIP on MPF inactivation. Histograms show histone H1 kinase activity monitored at 30, 60 and 90 min from the beginning of insemination (A) or ethanol treatment (B) in oocytes previously exposed to 20 µmol/l myr-AIP. Data were expressed as mean ± SEM of ratios of band intensity in the experimental groups to that of untreated metaphase II arrested oocytes. Different superscripts indicate statistical differences at $P < 0.05$. CaM kinase II is activated in mouse oocytes at fertilization
kinase II is stimulated in response to an explosive Ca$^{2+}$ rise like that occurring 1–3 min after sperm–oocyte fusion (Lawrence et al., 1997). However, the presence of a second increase of CaM kinase II activity seems to be peculiar to fertilized oocytes since it was not observed in oocytes artificially activated by ethanol through a monotonic Ca$^{2+}$ signal. This strongly suggests that CaM kinase II, after initial transient activation, is stimulated further by the succeeding Ca$^{2+}$ oscillations.

To explain the present results, one must take into account either the pattern of Ca$^{2+}$ oscillations in mouse oocytes or the regulatory properties of CaM kinase II. In monospermic mouse oocytes, Ca$^{2+}$ oscillations begin 7–19 min after the end of the first rise, are shorter than the initial spike and proceed at intervals of 20–30 min with some variability among individual oocytes (Deguchi et al., 2000). Moreover, as the oocytes progress along meiosis the intervals between Ca$^{2+}$ transients are prolonged and their duration becomes shorter. Under our experimental conditions, where synchronous fertilization is associated with a certain degree of polyspermy, the mean early frequency of Ca$^{2+}$ oscillations presumably increases slightly, as previously reported (Faure et al., 1999). Concerning CaM kinase II regulatory features, it is known that once activated by an increase in Ca$^{2+}$ calmodulin level, the kinase activity falls when Ca$^{2+}$ calmodulin returns to base levels. However, as a result of autophosphorylation, some subunits of the enzyme can remain active for a period of time prior to inactivation by phosphatases (Schulman and Hanson, 1993). This condition enables the kinase to increase the response to low frequency subthreshold stimuli, probably because autophosphorylation is functionally co-operative (Hanson and Schulman, 1992).

Our data suggest that, in mouse oocytes, the first sperm-induced Ca$^{2+}$ spike, as well as an artificial monotonic Ca$^{2+}$ increase, represent a threshold stimulus for kinase activation. Thus, it is conceivable that once Ca$^{2+}$ drops to base levels, CaM kinase II decreases its activity. Immunofluorescence studies on artificially activated mouse oocytes indicate that such a stimulus may cause, in a given cellular compartment, the maintenance of a low level of CaM kinase II activity not detectable in the biochemical assay used (Hatch and Capco, 2001). Given this state of activity, subsequent Ca$^{2+}$ oscillations following the initial spike may act as subthreshold stimuli that induce CaM kinase II to gradually increase its biochemical activity and produce a late peak, which under our experimental conditions is detectable at 60 min of insemination. According to this hypothesis, it is likely that, in monospermic oocytes, as a result of a Ca$^{2+}$ oscillation frequency lower than that of polyspermic oocytes, reduced levels of CaM kinase II activity could be detected at the time points investigated in the present study.

All the results reported above show that, in mouse oocytes, variations in CaM kinase II activity occur after sperm fusion over a period during which Ca$^{2+}$-dependent events such as meiosis resumption and CG exocytosis occur. Thus, in the second part of this study, we tested the hypothesis that CaM kinase II may play a role as an effector of the fertilization Ca$^{2+}$ signal towards elements that control these events. In amphibian oocytes, CaM kinase II is known to control exit from M phase by activating the ubiquitin-dependent cyclin degradation pathway in response to the fertilization Ca$^{2+}$ signal (Lorca et al., 1994; Morin et al., 1994) and also by acting on c-mos degradation (Lorca et al., 1991, 1993). Here we have shown that, in mouse oocytes, inhibition of CaM kinase II, achieved by cell treatment with a specific antagonist, negatively affects the decrease in MPF activity and the release from metaphase arrest that occurs upon fertilization or ethanol exposure. Our results on artificially activated oocytes confirmed those from Johnson et al. who demonstrated that AIP inhibits CaM kinase II and prevents meiosis resumption in A23187-treated oocytes (Johnson et al., 1998).

![Figure 5. Effect of myr-AIP on meiosis resumption. Left, histograms showing the rate of meiosis resumption after 30, 60 and 90 min from the beginning of insemination (A) or ethanol treatment (B). Right, representative fluorescence micrographs of oocytes stained with 10 µg/ml Hoechst 33342: (A) Untreated fertilized oocytes at anaphase (a) and telophase stage (b); fertilized oocytes preloaded with 20 µmol/l myr-AIP at metaphase (c) and telophase (d) stage; arrowheads points to decondensed sperm heads. (B) Untreated ethanol-activated oocytes at anaphase (e) and telophase (f) stage; oocytes exposed to ethanol after treatment with 20 µmol/l myr-AIP at metaphase stage (g,h). Bar = 20 µm. Data in each bar represent the mean ± SEM of at least three experiments and 130–150 oocytes examined. Asterisk indicates statistical differences at P < 0.05.](image-url)
CaM kinase II is activated in mouse oocytes at fertilization

These findings also support the observation that when CaM kinase II is inhibited, both MPF inactivation and meiosis resumption are prevented in spite of the presence of a normal ethanol-induced Ca\(^{2+}\) signal. Altogether, these observations provide evidence that this kinase may play a pivotal role in regulating meiosis resumption in mammalian oocytes.

Nevertheless, in the present study, when AIP was applied prior to insemination, it was not able to cause a persistent inhibition of MPF inactivation and meiosis resumption, as in ethanol-activated oocytes. In particular, we showed that this CaM kinase II antagonist blocks both the decrease in H1 kinase activity and the resumption of meiosis occurring in fertilized oocytes within 30 min of insemination. However, at 60 min AIP no longer inhibited these events even though CaM kinase II activity was still at a low level. Thus, it seems that reduced levels of CaM kinase II activity in fertilized oocytes result in a delayed activation of the molecular mechanisms which lead to a normal metaphase–anaphase transition. These observations are consistent with the results from a previous study showing that the decrease in MPF activity that normally occurs upon fertilization is significantly delayed when calmodulin-dependent steps are presumed to be blocked by exposing mouse oocytes to a calmodulin antagonist (Xu et al., 1996). In this case, the view could be taken that the physiological role of CaM kinase II in controlling meiosis resumption is to regulate the timing of the mammalian oocyte exit from metaphase, as also suggested by a mathematical model (Dupont, 1998). However, one cannot rule out that the transient effect of CaM kinase II inhibition on meiosis resumption versus a total block, such as that observed in ethanol-activated oocytes, may be due to a lower inhibitory action of myr-AIP on CaM kinase II in fertilized oocytes where the kinase is subjected to a repetitive Ca\(^{2+}\) stimulus. Alternatively, in fertilized oocytes, it is reasonable to hypothesize that release from metaphase arrest is also controlled by other signalling pathways.

Numerous studies on mammalian oocyte activation have demonstrated the involvement of protein kinase C (Colonna and Tatone, 1993; Capco, 2001), a cytoplasmic signalling molecule that is activated upon fertilization or artificial activation (Gallicano et al., 1997). The observation that protein kinase C agonists are able to induce release from metaphase arrest in the absence of a Ca\(^{2+}\) signal and thus of a CaM kinase II-dependent step suggests that this event is under the control of various key signalling pathways (Colonna et al., 1997). A model for mammalian oocytes can be proposed where both protein kinase C and CaM kinase II are two pivotal signalling agents that act very early after fertilization and need to interact in order to ensure a proper initiation of a programme of early development. As proposed by Capco, the meiotic spindle, as a stable architectural element, may provide an important site where components of the various pathways interact.
co-localize in order to increase the efficiency of the reactions involved in the release from metaphase arrest (Capco, 2001).

Emerging studies indicate that competence of the oocyte to undergo full activation is associated with its ability to generate Ca\(^{2+}\) oscillations (Ozil and Swann, 1995; Lawrence et al., 1998). A monotonic Ca\(^{2+}\) increase, such as that caused by most parthenogenetic agents, is adequate to stimulate the completion of meiosis only in aged oocytes where meiosis arresting factors have spontaneously decreased since ovulation (Jones, 1998). It has been proposed that Ca\(^{2+}\) oscillations are needed to maintain a low activity of MPF, once it has been reduced by the first large Ca\(^{2+}\) rise (Collas et al., 1993, 1995). On the basis of our results, this effect may be mediated by CaM kinase II, a Ca\(^{2+}\) effector whose activity appears to be differentially modulated in mouse oocytes depending on whether the Ca\(^{2+}\) rise is monotonic or repetitive.

In the present study, we propose an additional role for CaM kinase II in the oocyte activation process, which concerns the regulation of CG exocytosis, as previously suggested by direct and indirect evidence in mouse oocytes (Tatone et al., 1999; Abbott et al., 2001). Our results show that when CaM kinase II is inhibited, fertilized oocytes failed to complete the exocytosis process and this provides the first evidence for a physiological role of this kinase in this process. Since under our experimental conditions a complete inhibition of CG loss was not achieved, it cannot be ruled out that other signalling molecules participate in the control of this process. A candidate for this role is protein kinase C, even though its physiological involvement in CG exocytosis is still unclear. In somatic cells, CaM kinase II and protein kinase C are thought to participate in the secretion process by regulating different steps (Greengard et al., 1993). Of particular interest is the ability of CaM kinase II to phosphorylate synapsin-1, an event that regulates vesicle translocation, and components of the fusion machinery, such as certain SNARE proteins and rabphilin-3A, a known effector of the small GTP-binding proteins (Rabs) that regulate membrane trafficking events (Kato et al., 1994; Sudhoff, 1995; Gonzalez and Scheller, 1999; Watson, 1999). Moreover, the SNARE protein SNAP-25, and rabphilin-3A and Rab 3A localize to the cortex of mouse oocytes (Masumoto et al., 1998; Masumoto et al., 1998), an area where CaM kinase II has been detected by immunofluorescence (Johnson et al., 1998), and a role for rabphilin-3A as an important Ca\(^{2+}\) effector in the regulation of CG exocytosis has been proposed (Masumoto et al., 1998).

From the present results, there are at least three kinds of evidence to support our working hypothesis that CaM kinase II acts as an effector of the fertilization Ca\(^{2+}\) signal: (i) it is physiologically activated at fertilization; (ii) it is essential to trigger normal oocyte activation; (iii) its activation seems to be differentially modulated by a monotonic or repetitive Ca\(^{2+}\) rise. Even though further research is needed to investigate its behaviour during the late events of oocyte activation, the current investigation supports a model in which CaM kinase II is an upstream-acting component of the Ca\(^{2+}\) response machinery that senses calcium oscillations and disperses these signals to elements that control the cell cycle and exocytosis.

This investigation elucidates the Ca\(^{2+}\) response pathway in mammalian oocytes and helps to understand the functional significance of the fertilization Ca\(^{2+}\) signals. Results in this field are relevant to human IVF programmes since poor development of fertilized oocytes has been associated with non physiological Ca\(^{2+}\) signals related to oocyte immaturity or cytoplasmic sperm injection (Herbert et al., 1997; Tesarik, 1998).

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### References


