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Fertilization stimulates long-lasting oscillations of CaMKII activity in mouse eggs

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Abstract

Elucidation of the biochemical mechanisms by which specific proteins transduce the all important intracellular calcium (Ca^{2+}) signal at fertilization into events of egg activation will increase our understanding of the regulation of the onset of development and the extent to which these signals can be experimentally modified. Previously, we reported data supporting the hypothesis that mouse eggs have the capability to generate oscillations of the activity of Ca^{2+} and calmodulin-dependent kinase II (CaMKII), regulating the cell cycle and secretion. This study directly demonstrates transient increases of enzyme activity in relatively close synchrony with Ca^{2+} oscillations for the first hour of fertilization in single mouse eggs monitored for both Ca^{2+} and CaMKII activity. The extent of the enzyme activity increase was correlated with the level of intracellular Ca^{2+}. After a rise in activity, the decrease in activity did not appear to be due to negative feedback from elevated Ca^{2+} or CaMKII activity over time, since enzyme activity persisted after 8 min of elevated Ca^{2+} from 7% ethanol activation. The contribution of CaMKII from a single sperm to the rise in CaMKII activity at fertilization appeared to be negligible. Also, long-term cell cycle inhibition was observed in fertilized eggs with the CaMKII antagonist myrAIP (50 μM), which did not inhibit the first large Ca^{2+} transient or subsequent early oscillations but did reduce the percentage of eggs fertilized. Thus, mammalian eggs appear to drive many activation events over time to completion with repeated short bursts of Ca^{2+} oscillation-dependent CaMKII activity, rather than by a steady-state, continuously elevated level of CaMKII activity that is maintained by periodic Ca^{2+} oscillations.

Keywords: Egg; Intracellular calcium; CaMKII; Cell cycle; Meiosis; Fertilization

Introduction

A great deal more is known about the calcium (Ca^{2+}) dependence of the events of fertilization than the mechanism by which proteins transduce the Ca^{2+} signal into biological events. After fertilization of the mouse egg, cortical granule (CG) exocytosis and cell cycle progression are dependent upon Ca^{2+} and calmodulin (CaM)-dependent kinase II (CaMKII) (Tatone et al., 2002), which is present in mouse eggs and whose activity increases upon fertilization or parthenogenetic activation (Abbott et al., 2001; Hatch and Capco, 2001; Johnson et al., 1998; Markoulaki et al., 2003; Tatone et al., 1999, 2002; Winston and Maro, 1995). However, the pattern of CaMKII activity stimulated by long-lasting Ca^{2+} signals in the mammalian egg and the relationship of this activity to the events of egg activation have not been elucidated.

In mammalian fertilization, Ca^{2+} dependence is known for CG exocytosis (and the polyspermy block), exit from metaphase of meiosis II, entry into interphase, and recruitment of maternal mRNAs (Fissore et al., 2002; Runft et al., 2002; Schultz and Kopf, 1995). Ca^{2+} signaling is oscillatory with approximately several minutes of duration for individual transients, 5–30 min frequency, and multi-hour over-all duration, with species specific characteristics (Jones, 1998; Miyazaki et al., 1993; Swann and Ozil, 1994). In parthenogenetic studies, manipulation of Ca^{2+} oscillation parameters has significant effects on both pronuclear formation and much later post-implantation development (Ozil, 1990; Ozil

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and Huneau, 2001; Vitullo and Ozil, 1992), the progress of individual events of egg activation (Cran et al., 1988; Lawrence et al., 1998; Markoulaki et al., 2003; Xu et al., 1996), and egg viability (Gordo et al., 2000). Moreover, meiotic progression is Ca^{2+}-amplitude dependent in fertilized eggs (Nixon et al., 2002), and individual egg activation events are dependent upon the number of experimentally induced Ca^{2+} pulses (Ducibella et al., 2002).

Ca^{2+} oscillation parameters are decoded by CaMKII into changes in enzyme activity and autophosphorylation, from studies of the enzyme in vitro (De Koninck and Schulman, 1998; Hudmon and Schulman, 2002). High (ms) frequency Ca^{2+} oscillations result in sustained enzyme activity due to autophosphorylation (so-called autonomous activity), whereas low (s/min) frequency is likely to generate transient increases and decreases in activity. Although the approximately 5- to 15-min frequency of the fertilized mouse egg is expected to result in many hours of oscillatory CaMKII activity, this prediction is subject to many factors in vivo, including phosphatase activities (Bradshaw et al., 2003; Easom et al., 1998), ATP/ADP levels (Kim et al., 2001), and endogenous inhibitors (Chang et al., 2001) that have been reported in other cells.

Recently, we began to investigate the hypothesis that CaMKII activity oscillates in mouse eggs by stimulating two ionomycin-induced Ca^{2+} transients with fertilization-like frequency. Enzyme activity increased and decreased approximately in synchrony with intracellular Ca^{2+} levels (Markoulaki et al., 2003). By monitoring the same eggs for Ca^{2+} and CaMKII, it was also shown that the first fertilization-induced Ca^{2+} increase was accompanied by a rise in CaMKII activity, which began to decrease 1 min after Ca^{2+} levels began to decline.

Those experiments raised several questions that we attempted to answer in this study. (i) Does CaMKII activity return to basal levels after the first fertilization-induced Ca^{2+} rise? (ii) What is the pattern of CaMKII activity after multiple Ca^{2+} oscillations? (iii) What is the correlation between the levels of Ca^{2+} and CaMKII activity? and (iv) Is the inactivation of the enzyme mediated by a feedback signal from a "sustained" rise in Ca^{2+} and CaMKII activity? Finally, although myristoylated autocamtide-2-related inhibitory peptide (myrAIP), a specific inhibitor for CaMKII (Ishida et al., 1995), has been used successfully to inhibit CG exocytosis and exit from metaphase of meiosis II, the effect was only transient in fertilized eggs (Tatone et al., 2002). One of the aims of this study was to determine whether more complete inhibition could be obtained.

Materials and methods

Egg collection

Ovulated metaphase-II (MII)-arrested eggs were obtained from 6- to 14-week-old female CF-1 mice (Charles River Laboratories, Wilmington, MA) following injections of 6 IU pregnant mare's serum gonadotropin (Calbiochem, San Diego, CA) and, 48 h later, 6 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, MO). Cumulus masses were collected 13–14 h post-hCG in Earles Balanced Salt Solution (EBSS; Sigma) supplemented with 25 mM HEPES buffer at pH 7.3 and 0.3% BSA (Gibco-BRL, Gaithersburg, MD), at 35 °C. Cumulus cells were removed using 0.015% hyaluronidase (Calbiochem) in EBSS for 2–4 min, and zonae were dissolved with Tyrode's solution (pH 2.7; exposure <1 min) (Hogan et al., 1986). Before use, eggs were allowed to recover at least 30 min in 5% CO_2 at 37 °C in 1 ml of Minimal Essential Medium (MEM) (Eppig, 1999) containing 1% BSA (Sigma).

In vitro fertilization (IVF) and Ca^{2+} monitoring

Sperm from the cauda epididymides of 25- to 28-week-old CF-1 male proven breeders (Charles River) were collected in MEM with 1% BSA and allowed to capacitate at least 1 h at 37 °C, 5% CO_2. Eggs were loaded intracellularly with the fluorescent Ca^{2+} indicator Fura-2 AM (0.75 μM), adhered with CellTak® (Mehlmann and Kline, 1994), inseminated, and monitored for Ca^{2+} as previously described (Markoulaki et al., 2003) using a 10× Super-Fluor objective (Nikon, Tokyo, Japan), a Nikon Eclipse TE2000-U inverted fluorescent microscope, and IP Lab software (version 3.6.3 with RatioPlus package; Scanalytics, Fairfax, VA).

Ethanol- and cycloheximide-mediated egg activation

Zona-free eggs were placed in 7% ethanol (EtOH) in EBSS supplemented with 0.3% BSA, and cultured for 2 or 8 min at 35 °C before being washed in EtOH- and Ca^{2+}/Mg^{2+}-free EBSS and frozen for CaMKII activity assays (see below).

Zona-free eggs were placed in 0.5 ml MEM containing 0.1% PVA and parthenogenetically activated using 10 μg/ml cycloheximide (Sigma) (Moos et al., 1996) in the absence or presence of 50 μM myrAIP. Eggs were examined at 2 h and every 30 min thereafter until 5.5 h post-activation.

Freezing eggs and sperm for CaMKII activity assays

A single egg fertilized in vitro, or three eggs treated with EtOH, were rapidly washed in Ca^{2+}/Mg^{2+}-free EBSS and transferred into a microfuge tube for CaMKII activity assays as previously described (Markoulaki et al., 2003). After transferring the egg(s) into 2 μl lysis buffer (total volume frozen approximately 2.5 μl), the tube was immediately submerged into liquid nitrogen for 5 s before being placed in dry ice and stored at −80 °C.

Sperm from the cauda epididymides of male proven breeders were allowed to capacitate (above) and transferred to a 500-μl thin-wall PCR tube (USA Scientific, Ocala, FL). The tube was centrifuged for 10 min at low speed, and 1×10^5

sperm/ml (0.5 μl) were transferred into 2 μl of lysis buffer in a tube, which was placed on dry ice and stored at −80 °C.

CaMKII activity assays

Two types of enzyme activity, autonomous and maximal, were analyzed as previously described (Markoulaki et al., 2003). Briefly, "autonomous activity", representing autophosphorylated enzyme that remains active in the absence of Ca^{2+}/CaM was examined in the absence of exogenous Ca^{2+}/CaM in the reaction mixture, whereas maximal activity was examined in the presence of excess Ca^{2+}/CaM. Unless otherwise indicated, CaMKII activity refers to autonomous activity. The SignaTECT assay system (Promega, Madison, WI) utilizing a biotinylated peptide substrate and 2.5 μCi [γ^{32}P]ATP (Perkin Elmer, Boston, MA) was used as previously described (Markoulaki et al., 2003).

DNA staining

Inseminated eggs were fixed and stained for DNA using DAPI (Polysciences, Warrington, PA) and Hoechst 33258 (Sigma) to determine the cell-cycle and fertilization status as previously described (Ducibella et al., 1994). Fertilization was indicated by the presence of decondensed sperm head chromatin.

IVF in the presence of myrAIP

To prevent a decrease in the effective concentration of myrAIP, both oil and albumin were omitted from the insemination medium (e.g., oil overlay was found to remove inhibitory activity). Albumin was used for sperm capacitation and PVA replaced it in the MEM medium. For determining effects on the cell cycle, zona-free eggs in 0.5 ml MEM containing myrAIP (Biomol, Plymouth Meeting, PA) and 0.1% PVA (Sigma) were inseminated with 1.5×10^5 sperm/ml (final concentration) at 37 °C, 5% CO_2 in a 4-well Nunclon Δ multidish (Fisher Scientific, Pittsburgh, PA). A 10-mM stock solution of myrAIP was made on the morning of the experiment using embryo-culture quality water (Sigma).

For Ca^{2+} imaging, treated eggs were pre-incubated in 50 μM myrAIP in 0.5 ml MEM containing 0.1% PVA at 37 °C, 5% CO_2 for 1 h before insemination. Ten to 20 zona-free eggs were adhered with Cell-Tak® to an 8-well Nunc Lab-Tek Chambered Cover Glass (Fisher Scientific), inseminated with approximately 5×10^4 sperm/ml in 0.5 ml MEM containing 0.1% PVA in the presence or absence of 50 μM myrAIP, and monitored for Ca^{2+} (see above). Heated, humidified 5% CO_2 in air was applied to the chamber to maintain pH.

Statistics

CaMKII activity for high and basal levels of Ca^{2+} was compared using the unpaired Student's *t* test. Second polar body extrusion in the absence or presence of myrAIP in fertilized eggs was compared using the chi-square (χ^2) analysis.

Results

CaMKII activity oscillates for at least 1 h during fertilization

In a previous report, it was shown that CaMKII activity increases during the first fertilization-associated rise in intracellular Ca^{2+} and decreases 1 min after Ca^{2+} has dropped to basal levels, suggesting that CaMKII may oscillate during the early stages of fertilization. However, the possibility that enzyme activity progressively increases over time until it reaches a plateau could not be excluded and was investigated during the first hour of fertilization.

Fertilized eggs were identified by their characteristic increases in intracellular Ca^{2+} by Fura-2 monitoring. Individual eggs were frozen at different Ca^{2+} levels to examine relative changes in CaMKII activity in response to Ca^{2+} on a single egg basis. Activity was determined in single eggs frozen before, during, and after both the first and third Ca^{2+} oscillation, as well as during and after the oscillation 1 h post-insemination (Fig. 1A, arrows). The figure and text present mean activity values. Classification of the groups of eggs, as well as the rationale for choosing the groups, is shown in Table 1.

CaMKII activity (Fig. 1B) exhibited an increase of 163% during the peak of the first fertilization-associated increase in intracellular Ca^{2+} (Peak I) as compared to untreated eggs. Also, as previously observed (Markoulaki et al., 2003), 1 min after Ca^{2+} had reached (post-peak) basal levels, CaMKII activity decreased by approximately 50% (Baseline I). We now show that by 2 min following the drop in Ca^{2+} (Baseline I′), CaMKII activity fell dramatically further to approximately 15% of its observed peak value. It should be noted that as in other studies (Miyazaki et al., 1993), the baseline Ca^{2+} level can be slightly higher after the first Ca^{2+} transient than before. Before the third Ca^{2+} oscillation, CaMKII activity was relatively low (Baseline II), but it increased rapidly, 110%, in response to the next Ca^{2+} rise (Peak III). Two minutes after the third transient, the enzyme activity returned to a low level (Baseline III).

To avoid long-term monitoring artifacts, eggs that had initiated Ca^{2+} oscillations for approximately 20–30 min were placed in the incubator. Thirty min later (approximately 1 h post-insemination), the exact number of Ca^{2+} oscillations undergone by each egg was not known. However, based on the initial Ca^{2+} oscillation frequency of the same eggs, it was estimated that an average of 11 oscillations had taken place. At 1-h post-insemination, CaMKII activity appeared to continue to oscillate in response to Ca^{2+}, exhibiting a 140% increase at/near peak levels of Ca^{2+} and basal levels of activity 2 min after the drop in Ca^{2+} (Fig. 1B). Eggs were

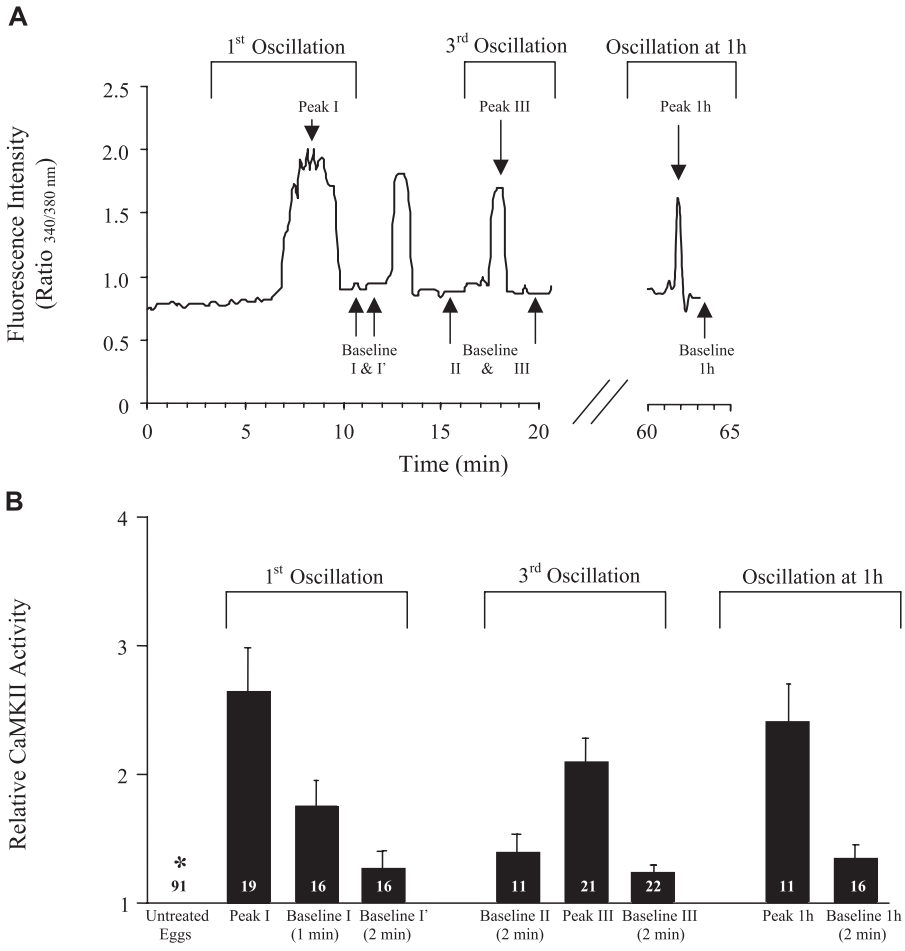


Fig. 1. CaMKII activity on a single-egg basis during the first hour of IVF. (A) Representative composite Ca^{2+} trace for 1 egg. Ca^{2+} monitoring was initiated within 1 min of insemination of four to five eggs. The CaMKII activities of single eggs were examined at the Ca^{2+} levels indicated by arrows. When an egg had reached a Ca^{2+} level designated in the figure, that egg was detached from the culture dish (without stopping Ca^{2+} monitoring) and frozen within 30 s for CaMKII analysis (Markoulaki et al., 2003; Materials and methods). The remaining eggs were frozen later after reaching a designated Ca^{2+} level. For eggs frozen 1 h post-insemination, Ca^{2+} monitoring was performed until the end of the third Ca^{2+} oscillation (approximately 20–30 min), and then eggs were placed in a CO_2 incubator for approximately 30 min of culture (rationale in Table 1 footnote "i"). At 1 h post-insemination, monitoring was re-initiated and eggs were frozen at the peak of Ca^{2+} or 2 min after Ca^{2+} had returned to basal levels (Table 1). The gap in the Ca^{2+} trace between 20 and 60 min post-insemination represents the time during which the eggs were in the incubator. Since fertilization was observed within 10 min of insemination, the state of fertilization before culture in the incubator was known and recorded for each egg. Furthermore, due to reversible immobilization with CellTak®, specific eggs were identified by location after re-initiation of monitoring. Fertilized eggs were frozen at 1 h. (B) Mean CaMKII activity values from single eggs frozen at the Ca^{2+} levels indicated in (A). *CaMKII activity, in these and all later experiments, is expressed relative to the activity present in untreated metaphase II eggs, which was set at 1. Values represent the mean ± SEM (standard error of the mean). Peak values are statistically different than their corresponding Baseline values (*t* test; $P < 0.01$). The number of determinations (1 egg/determination) is shown at the base of each bar. Unless otherwise indicated, these experiments and subsequent ones were performed at least three times. These experiments required two people.

not frozen before the Ca^{2+} peak at 1 h because it would not have been known if the egg had stopped oscillating while in the incubator (a false-negative result) or if a Ca^{2+} rise had been imminent and would have occurred after monitoring and before freezing (a false-positive).

Comparison of CaMKII activity in sperm and eggs

Capacitated sperm exhibit detectable levels of CaMKII activity. One "sperm-equivalent" of (autonomous) activity and maximal activity are 0.004% and 0.01%, respectively, compared to the mean level for an unfertilized egg assayed for autonomous activity. Each activity assay, autonomous and maximal, included three sperm determinations (1×10^5 sperm each) and three egg determinations (3 eggs/determination). The assays were repeated twice.

The levels of CaMKII activity and intracellular Ca^{2+} are correlated

The relationship between the relative levels of intracellular Ca^{2+} and CaMKII activity was investigated by plotting mean enzyme activity versus increasing levels of the 340/380 nm ratio for eggs under different stimulatory conditions (Fig. 2). An elevation of relatively low amplitude, such as that of the third fertilization-induced Ca^{2+} oscillation, resulted in a

Table 1
Classification of eggs for CaMKII activity assays

Group	Ca^{2+} oscillation number[a]	Insemination[b]	Ca^{2+} levels[c]	Timing of egg detachment[d] (approximately 30 s before freezing)
Untreated	–	No	Basal	Before insemination
Peak I	1	Yes	High	approximately 2 min after Ca^{2+} increase onset[e]
Baseline I	1	Yes	Basal	approximately 1 min after the Ca^{2+} decrease
Baseline I′	1	Yes	Basal	approximately 2 min after the Ca^{2+} decrease[f]
Baseline II	2[g]	Yes	Basal	approximately 2 min after the 2nd Ca^{2+} decrease[f]
Peak III	3	Yes	High	approximately 1/3 – 1/2 of total Ca^{2+} increase[h]
Baseline III	3	Yes	Basal	approximately 2 min after the Ca^{2+} decrease[f]
Peak 1 h	approximately 11[i]	Yes	High	approximately 1/3 – 1/2 of total Ca^{2+} increase[h]
Baseline 1 h	approximately 11[i]	Yes	Basal	approximately 2 min after the Ca^{2+} decrease[f]

[a] From detachment to the time of egg freezing.
[b] 5×10^4 sperm/ml.
[c] Based on the ratio of 340/380 nm. "Basal" includes small changes in the Ca^{2+} baseline. Representative Ca^{2+} levels for each group of eggs are shown in Fig. 1A (arrows).
[d] Detachment of egg from the Ca^{2+}-monitoring chamber immediately before freezing via liquid N_2.
[e] Since the first Ca^{2+} elevation lasts for 3–4 min, eggs were frozen approximately 2 min after the onset of the Ca^{2+} rise.
[f] Based on previous observations (Markoulaki et al., 2003), CaMKII activity is still elevated at 1 min after the onset of the decrease in Ca^{2+} at the end of the first Ca^{2+} transient. To determine whether enzyme activity returns to resting levels, eggs were frozen 2 min after Ca^{2+} had returned to basal levels.
[g] Basal levels of Ca^{2+} before the 3rd oscillation (Fig. 1A).
[h] Since later Ca^{2+} rises have a much shorter duration (≤1 min) than the first, eggs at high Ca^{2+} levels during the third and "1 h" oscillations (Fig. 1A) were frozen as soon as an unequivocal Ca^{2+} rise had started but before it had reached maximum levels (in most cases 1/3–1/2 of the maximum Ca^{2+} rise, estimated from the amplitude of the second oscillation of that egg).
[i] This number represents an estimate of the average number of Ca^{2+} oscillations undergone by the eggs approximately 1 h post-insemination, based on their frequency during the first 20–30 min post insemination. The precise number of oscillations undergone by each egg 1 h post-insemination requires continuous Ca^{2+} monitoring. Since it affects the pH and temperature of the culture medium, continuous monitoring was not performed (Fig. 1).

relatively small increase in CaMKII activity (110% compared to untreated eggs), whereas a higher amplitude Ca^{2+} rise, such as that of the first Ca^{2+} oscillation, resulted in higher enzyme activity (163%). An ionomycin-induced rise (Markoulaki et al., 2003), which is larger compared to that of the first fertilization-associated Ca^{2+} oscillation (340/380 nm ratios of 4.0–5.0 and 2.0–3.0, respectively), resulted in a mean increase of 307% in CaMKII activity (Fig. 2). Finally, when egg lysates were assayed for enzyme activity in the presence of excess exogenous Ca^{2+} and CaM, the levels of CaMKII activity were highest with a mean increase of 900% over lysates in their absence.

Prolonged Ca^{2+} and CaMKII activity do not cause CaMKII inactivation

After a transient increase in enzyme activity, the mechanism of CaMKII inactivation in the mammalian egg is

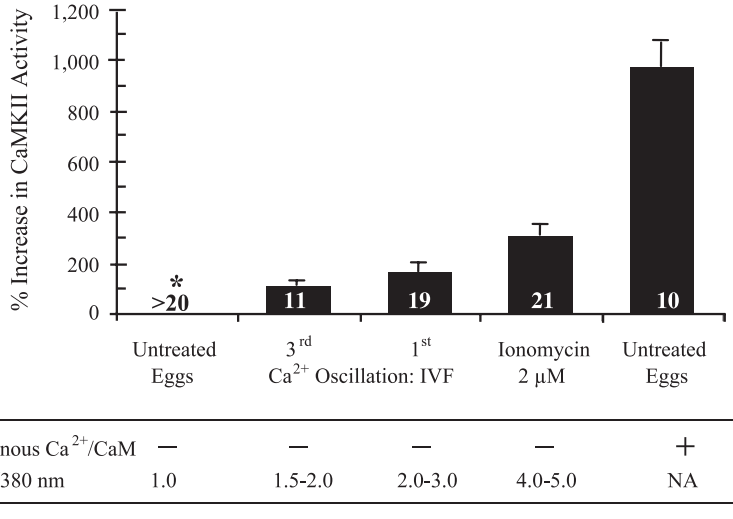


Fig. 2. The level of CaMKII activity is directly correlated to the level of Ca^{2+}. The groups on the *x*-axis are in order of increasing Ca^{2+} levels as shown by the 340/380 nm ratio. A 340/380 nm value is not applicable (NA) in untreated eggs in the presence of excess Ca^{2+}/CaM because lysates were used. Bars represent mean ± SEM, and the number of determinations is shown at the base of each bar (IVF oscillations: 1 egg/determination; ionomycin and untreated eggs: 5 eggs/determination).

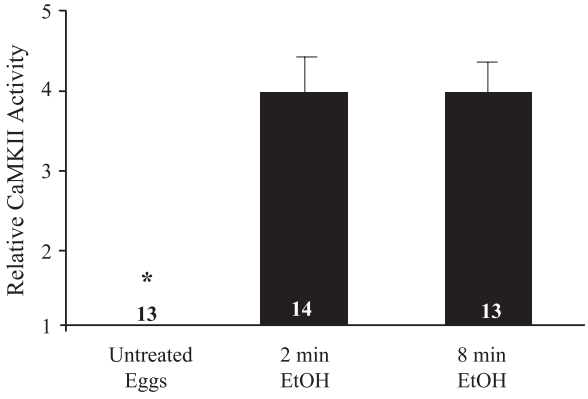


Fig. 3. CaMKII inactivation is not mediated by a sustained increase in Ca^{2+} or enzyme activity after treatment with 7% EtOH. Bars represent the mean ± SEM, and the number of determinations (3 eggs/determination) is shown at the base of each bar.

currently unknown. To test the hypothesis that enzyme inactivation is mediated by a negative feedback signal from a sustained increase in intracellular Ca^{2+} and/or CaMKII activity, a prolonged increase in intracellular Ca^{2+} (total of 8 min) was induced by parthenogenetically activating eggs with 7% EtOH. CaMKII activity was examined in eggs 2 and 8 min after the onset of EtOH treatment. The EtOH-induced increase in intracellular Ca^{2+} was synchronous and reproducible. CaMKII activity increased by a mean of 292% by 2 min after the onset of EtOH treatment compared to untreated eggs. At 8 min, CaMKII activity remained elevated (293%; Fig. 3).

CaMKII antagonist myrAIP inhibits cell cycle progression in fertilized, but not cycloheximide-treated, eggs

Inhibition of CaMKII activity using myrAIP has been shown to transiently prevent exit from metaphase of meiosis II in fertilized mouse eggs (Markoulaki et al., 2003; Tatone et al., 2002). MyrAIP is a myristolyated peptide based on the autoinhibitory domain of CaMKII (Ishida et al., 1995). To more completely investigate the effects of myrAIP on cell cycle progression, we determined (i) the dose–response curve for enzyme activity in the presence of myrAIP, for both purified brain enzyme and unfertilized egg lysates, and (ii) the incidence of second polar body (PB) extrusion and pronuclear (PN) formation in the presence or absence of the inhibitor.

A dose-dependent inhibition of CaMKII activity for both purified brain enzyme and egg lysates was observed in response to myrAIP (Fig. 4). The CaMKII activities of 0.1 ng purified enzyme (amount estimated in 1 metaphase II egg; (Markoulaki et al., 2003) and in the lysate of 1 metaphase II egg in the absence of myrAIP were considered as 100% activity. In the presence of 50 μM myrAIP, the activities of the purified enzyme and the egg lysate were inhibited by 90% and 80%, respectively (Figs. 4A and B).

In inseminated eggs, a dose-dependent inhibition of PB extrusion and PN formation was observed in the presence of myrAIP (Figs. 5E and F). Incubation in 50-μM myrAIP from the time of insemination resulted in 16% PB extrusion at 2 h after insemination (Figs. 5B and E) compared to 88% in its absence (Figs. 5A and E). To determine if the decrease was due to an effect of myrAIP on fertilization, living eggs were scored for % PB extrusion and then all eggs were examined by chromatin staining for a decondensing sperm head, indicative of fertilization. Interestingly, 50-μM myrAIP decreased the percentage of eggs fertilized by about one half (48% vs. 95% in controls). However, despite the lower fertilization rate, PB extrusion was still significantly inhibited in fertilized eggs treated with myrAIP compared to fertilized controls (Fig. 5G). This difference is apparent in fertilized eggs fixed at both 70 and 120 min post-insemination.

The effect of myrAIP on fertilization did not appear to be due to toxicity based on rescue experiments in which eggs removed from the inhibitor were able to undergo PB extru-

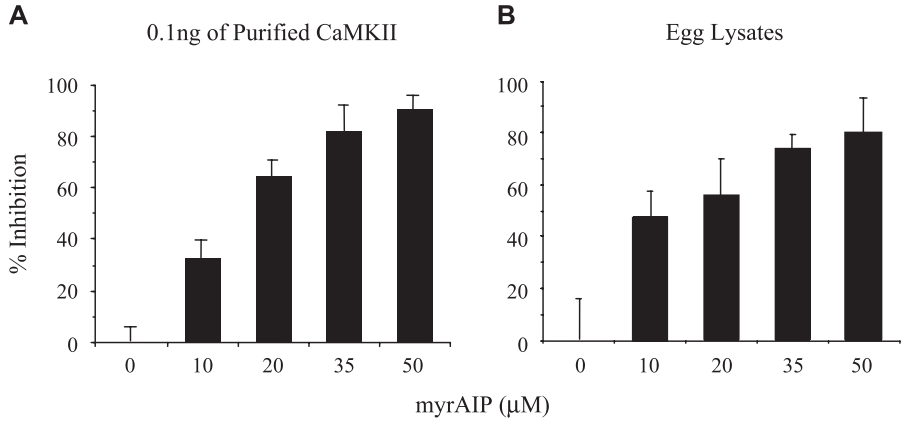


Fig. 4. The activities of purified CaMKII (A) and enzyme in egg lysates (B) are inhibited by myrAIP in a dose-dependent manner. Assays were conducted in the presence of excess Ca^{2+}/CaM (maximal activity, Materials and methods). Bars represent mean ± SEM. These experiments were performed twice with triplicates in each group.

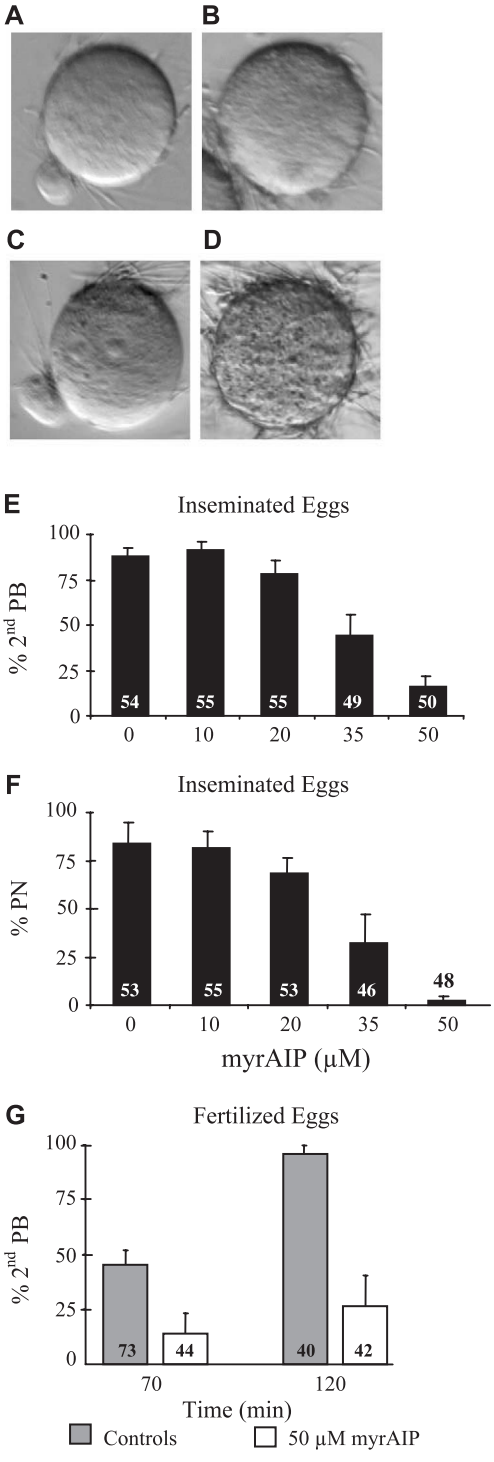


Fig. 5. Extrusion of the second PB and PN formation are inhibited in a dose-dependent manner by myrAIP. (A–D) Representative light micrographs of eggs at 2 h (A, B) or 7 h (C, D) post-insemination in the absence (A, C) or presence of 50 μM myrAIP (B, D). Inseminated eggs: (E) extrusion of PB (120 min) and (F) PN formation (7 h). (G) Fertilized eggs: percent of PB extrusion in the absence or presence of 50 μM myrAIP is significantly different (χ^2, $P < 0.005$). Fertilization (70 and 120 min post-insemination) was based on the presence of decondensed sperm head in stained eggs. Values represent mean ± SEM. The number of eggs analyzed is shown at the base or top of each bar.

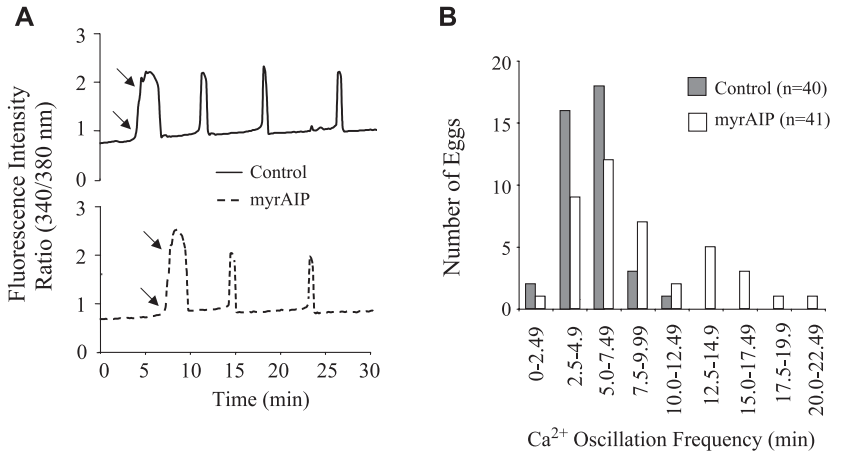
sion and PN formation (data not shown). A 50-μM myrAIP did not affect PB extrusion in uninseminated eggs treated with 10 μg/ml of cycloheximide (CHX) (Moos et al., 1996) for 5.5 h, suggesting that pathways downstream of cyclin B1 degradation were not inhibited by myrAIP [%PB: 39% + CHX (n = 33); 40% + CHX/myrAIP (n = 30); 7% no CHX/myrAIP (n = 41)].

CaMKII antagonist myrAIP does not inhibit Ca^{2+} oscillations

To address the possibility that inhibition of PB extrusion by myrAIP is mediated by preventing Ca^{2+} oscillations rather than a direct effect of the inhibitor on CaMKII, Ca^{2+} was monitored in the presence or absence of 50 μM myrAIP in fertilized eggs. To provide maximal inhibition in mouse eggs (Tatone et al., 2002) and simulate the cumulative exposure near the time of the onset of PB extrusion, eggs were preincubated for 1 h in myrAIP, inseminated, and monitored for up to 1 h. No major changes in the first large Ca^{2+} transient and subsequent smaller rises were observed (Fig. 6A). A distribution plot of Ca^{2+} oscillation frequency demonstrated that the majority of eggs treated with myrAIP had oscillation frequencies similar to those of untreated eggs; however, a small proportion of eggs exhibited prolonged frequencies with myrAIP (Fig. 6B). Although such an extended frequency might be expected to result in delayed or failed PB extrusion, this subpopulation is too small to account for myrAIP-mediated cell cycle inhibition observed in the majority of eggs (Fig. 5; a delay was not observed during the 7 h of monitoring for PBs). A detailed analysis of the Ca^{2+} response in the first large transient indicated similar mean responses for duration, amplitude, and initial as well as final rates of increase (Fig. 6C).

Discussion

Although fundamentally important, little is known about the mechanism by which oscillatory Ca^{2+} signals are transduced into the onset of mammalian development and the extent to which these signals can be experimentally modified in animal and therapeutic cloning. The demonstration of long-term pulsatile CaMKII activity in this study provides strong evidence that mammalian eggs drive some activation events over time to completion with repeated short bursts of Ca^{2+} oscillation-dependent enzyme activity rather than by a steady-state, continuously elevated level of activity that is maintained by periodic Ca^{2+} oscillations. The results also provide a potential explanation for the long period of time required by mammalian eggs to complete meiosis compared to some other lower species. We also demonstrate the sensitivity of CaMKII activity to Ca^{2+} in vivo, the predominance of egg versus sperm CaMKII, and the sensitivity of the completion of cell cycle events to a CaMKII antagonist.



C

Analysis of fertilization-induced Ca^{2+} oscillations in the presence or absence of 50 μM myrAIP

	Control			myrAIP		
	n	Mean (SD[a])	SEM	n	Mean (SD[a])	SEM
Duration[b, c]	47	2.77 (0.67)	0.10	43	2.74 (0.47)	0.07
Amplitude[b, d]	47	2.55 (0.47)	0.07	43	2.42 (0.40)	0.06
Initial Rate of Increase[b, e]	47	2.34 (0.85)	0.12	43	2.40 (0.83)	0.13
Final Rate of Increase[b, f]	47	1.08 (0.70)	0.10	43	1.18 (0.81)	0.12
Frequency[g]	40	5.25 (2.17)	0.34	41	8.61 (4.69)	0.73

[a] Standard Deviation.
[b] First Ca^{2+} transient.
[c] Time in min of increased level of Ca^{2+} over baseline.
[d] Maximum relative fluorescence intensity (340/380 nm).
[e] Change in 340/380 nm over 20 sec starting at the time of a ≥10% increase in level of Ca^{2+} over baseline (see **Fig. 6A**).
[f] Change in 340/380 nm over 20 sec ending at the time of the initial highest increase in level of Ca^{2+} (see **Fig. 6A**).
[g] Time in min between the highest Ca^{2+} levels of the second and third Ca^{2+} oscillations.

Fig. 6. Comparison of Ca^{2+} responses in the presence or absence of 50 μM myrAIP. In each experiment, both treated and untreated eggs were taken from the same common egg pool and inseminated with the same sperm preparation. (A) Representative 340/380 nm ratios over time after insemination, which was ≤ 1 min before the onset of Ca^{2+} imaging (Time = 0). Arrows are described below. (B) Comparison of Ca^{2+} oscillation frequencies for groups of eggs in the presence or absence of myrAIP. The majority of eggs in both groups have similar distributions of oscillation frequency. However, myrAIP-treated eggs have a small subpopulation with a slower frequency as indicated by the bimodal distribution. (C) Comparison of Ca^{2+} transient parameters, including the initial and final rates of increase in the 340/380 nm ratio (in A, lower and upper arrows, respectively, as described by Jones et al., 1995). Note that the higher mean frequency in the treated group is due to the bimodal distribution in B. See Results for more information.

Oscillatory CaMKII activity for the first hour of fertilization (and likely for the duration of Ca^{2+} oscillations) provides an elegant biochemical mechanism to temporally regulate CaMKII-dependent events of egg activation, such as CG exocytosis and completion of meiosis II (see Introduction). A previous study demonstrated that different numbers of Ca^{2+} rises are required for the initiation of different events of mouse egg activation and that more rises are required for completion, rather than initiation, of CG exocytosis, cell-cycle kinase changes, and cell cycle progression (Ducibella et al., 2002). A hierarchy was found in which more Ca^{2+} pulses were required for PN formation than exit from metaphase of meiosis II, and fewer still for the onset of CG exocytosis. These results are consistent with theoretical models of egg activation events driven by oscillatory Ca^{2+} signaling and CaMKII (Dupont, 1998).

The fact that each Ca^{2+} rise is now known to be transduced by CaMKII into a pulse of enzyme activity provides evidence that CaMKII is an "incremental driver" of early embryonic events that depend on its activity. We postulate that continuing pulses of enzyme activity stimulate both secretory activity until all CGs are released and CaMKII-dependent cell cycle signaling until the decision is made for entry into interphase (ultimately visualized by PN). Each pulse of enzyme activity may transiently reinitiate secretion and cell cycle signaling, or maintain them if they have not ceased since the last Ca^{2+} oscillation. Consistent with this, each Ca^{2+} pulse is associated with additional CG exocytosis (Ducibella et al., 2002). Regarding the

cell cycle, in the face of continued cyclin B1 synthesis, multiple Ca^{2+} transients are required for the decrease in the levels of cyclin B1 protein and MPF activity required for meiotic completion (Collas et al., 1995; Ducibella et al., 2002; Nixon et al., 2002). Since the downregulation of cyclin B1 and MPF is dependent upon CaMKII (Lorca et al., 1993; Tatone et al., 2002) and Ca^{2+} pulse number, a series of pulses of CaMKII activity appear to be required to drive down MPF activity to a low level compatible with interphase entry and PN formation. In fertilized mouse eggs, which vary in their individual Ca^{2+} responses, a large decrease in the level of cyclin B1 appears to require 3–7 oscillations (Nixon et al., 2002), which is similar to the 4–8 Ca^{2+} pulses reported from parthenogenetic stimulation by controlled electropermeabilization (Ducibella et al., 2002).

Oscillatory CaMKII activity may result in temporal information about when certain events of egg activation take place. Support for temporal regulation comes from the fact that species with faster Ca^{2+} oscillation frequencies reach the PN stage sooner (discussed in Markoulaki et al., 2003) and that Ca^{2+} oscillation parameters affect the rate of PN formation (Ozil, 1998; Vitullo and Ozil, 1992). One proposal for a temporal mechanism of regulation is as follows: if the onset of an event is governed by the time (length) and amount of enzyme activity, a specific number of pulses of activity are likely to be required for event initiation. Each transient of CaMKII activity and following period of reduced activity (before the next rise) represents a time interval governed by the Ca^{2+} oscillation frequency of fertilization, which varies among species. Ozil et al. have shown that summation of Ca^{2+} signals regulates parthenogenetic egg activation (Huneau et al., 2003). Extending this idea to CaMKII, if each pulse of enzyme activity was additive at the level of substrate phosphorylation, a CaMKII-dependent event would not be initiated until enough time had elapsed to include the required number of oscillations of activity for sufficient substrate phosphorylation. Regarding Ca^{2+} pulse number dependence of event completion (Ducibella et al., 2002), an event would not be completed until an appropriate number of additional transients of CaMKII activity had occurred. The extent to which this actually takes place remains to be established and is very likely influenced by other factors, such as phosphatase activity and protein turnover or production (e.g., continued cyclin B1 synthesis).

Amphibian and fish eggs are also arrested at the metaphase II stage, but normally undergo only a single Ca^{2+} rise at fertilization. However, this oscillation is on the order of 15 min in length compared to 1–2 min for each mammalian Ca^{2+} rise (except the first which is 3–4 min) (Stricker, 1999). It is interesting to note that 15 min of continuously elevated Ca^{2+} is roughly equivalent to 10–20 typical mammalian Ca^{2+} transients which are sufficient for most Ca^{2+}-dependent events of mouse egg activation (Ducibella et al., 2002). It should be mentioned that each 1–2 min transient is at its maximum amplitude for <10% of its duration and that low amplitude Ca^{2+} oscillations may not be adequate to drive cyclin B1 destruction (Nixon et al., 2002) or egg activation (Ozil and Huneau, 2001).

This study also begins to address other means by which the amount or activity of CaMKII may be regulated at fertilization. Since sperm are reported to contribute an important factor (upstream of Ca^{2+}) for mammalian egg activation (Dale et al., 1985; Saunders et al., 2002; Swann, 1990), we determined the maximal amount of CaMKII activity that a single sperm could contribute to the fertilized egg. Since it was <0.01% of the measured activity in the newly fertilized egg, egg CaMKII appears to be the predominant source of enzyme. However, individual sperm may vary in the amount of activity and sperm are known to also have CaM (Courtot et al., 1999; Jones et al., 1980) and CaMKIV (Wu et al., 2001); the latter's activity and potential role in the events of fertilization are unknown.

Prolonged elevated Ca^{2+} from ethanol activation did not appear to cause a termination of CaMKII activity (after 8 min exposure), for example, by a negative feedback process involving a Ca^{2+}-dependent phosphatase. Our results are consistent with the idea that CaMKII activity may be downregulated by a Ca^{2+}-independent phosphatase, such as PP1 (Menegon et al., 2002; Strack et al., 1997), and/or autodephosphorylation (Kim et al., 2001). Phosphatase activity has been reported in activated mouse eggs (Winston and Maro, 1999).

Extending previous studies (Introduction), we found that myrAIP inhibits, in a dose-dependent manner, both purified CaMKII as well as PB extrusion and PN formation. Our results are consistent with the observation in *Xenopus* eggs that Ca^{2+} and CaMKII are involved in cdc20-dependent APC/C activation (Lorca et al., 1998; Reimann and Jackson, 2002), which is necessary for sister chromatid separation in *Xenopus* egg extracts (Lorca et al., 1998) and cdk1/cyclin B1 inactivation for entry into interphase in yeast (Shirayama et al., 1999). Although the dose–response analysis of myrAIP demonstrated that 50 μM myrAIP was optimal for inhibition of enzyme activity and cell cycle progression, this treatment was associated with a reduction in the percentage of eggs fertilized and with a small subgroup of fertilized eggs whose oscillation frequency was decreased. The molecular basis for these interesting effects, as well as the actual concentration and localization of myrAIP inside the eggs, remain to be established.

Our results also have implications for procedures that experimentally alter the temporal pattern of intracellular Ca^{2+} release during mammalian egg activation. Egg CaMKII (autonomous) activity is extremely sensitive to increasing and decreasing levels of intracellular Ca^{2+}, and prolonging the Ca^{2+} rise leads to extended enzyme activity. Thus, altered Ca^{2+} oscillations in eggs activated by intracytoplasmic sperm injection (Kurokawa and Fissore, 2003) are likely to lead to corresponding changes in CaMKII activity. Also, procedures like $SrCl_2$, used in animal cloning (Kishikawa et al., 1999), that cause longer duration divalent cation oscillations (Swann and Ozil, 1994) may result in an

increase in the total duration of enzyme activity over time (depending on the effectiveness of Sr^{2+} substituting for Ca^{2+}). Since it is possible that oscillations of CaMKII activity encode temporal information (above), an agent like $SrCl_2$ may also result in a given amount of cumulative enzyme activity earlier than during normal fertilization. Our studies suggest the need to examine if different activation protocols affect the timing of egg activation events and whether timing is crucial to later normal development. It is well known that the extent of post-implantation development after parthenogenetic activation is dependent upon the pattern of experimentally administered Ca^{2+} oscillations (Ozil and Huneau, 2001).

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References

Abbott, A., Fissore, R., Ducibella, T., 2001. Identification of a translocation deficiency in cortical granule secretion in preovulatory mouse oocytes. Biol. Reprod. 65, 1640–1647.

Bradshaw, M., Kubota, Y., Meyer, T., Schulman, H., 2003. An ultrasensitive Ca^{2+}/calmodulin-dependent protein kinase II-protein phosphatase 1 switch facilitates specificity in postsynaptic calcium signaling. Proc. Natl. Acad. Sci. U. S. A. 100, 10512–10517.

Chang, B., Mukherji, S., Soderling, T., 2001. Calcium/calmodulin-dependent protein kinase II inhibitor protein: localization of isoforms in rat brain. Neuroscience 102, 767–777.

Collas, P., Chang, T., Long, C., Robl, J.M., 1995. Inactivation of histone H1 kinase by Ca^{2+} in rabbit oocytes. Mol. Reprod. Dev. 40, 253–258.

Courtot, A., Pesty, A., Lefevre, B., 1999. Calmodulin, gametes and fertilisation. Zygote 7, 95–104.

Cran, D., Moor, R., Irvine, R., 1988. Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate. J. Cell Sci. 91, 139–144.

Dale, B., DeFelice, L., Ehrenstein, G., 1985. Injection of a soluble sperm fraction into sea urchin eggs triggers the cortical reaction. Experientia 41, 1068–1070.

De Koninck, P., Schulman, H., 1998. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science 279, 227–230.

Ducibella, T., Duffy, P., Buetow, J., 1994. Quantification and localization of cortical granules during oogenesis in the mouse. Biol. Reprod. 50, 467–473.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R., Kopf, G., Fissore, R., Madoux, S., Ozil, J.-P., 2002. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. Dev. Biol. 250, 280–291.

Dupont, G., 1998. Link between fertilization-induced Ca^{2+} oscillations and relief from metaphase II arrest in mammalian eggs: a model based on calmodulin-dependent kinase II activation. Biophys. Chem. 72, 153–167.

Easom, R., Tarpley, J., Filler, N., Bhatt, H., 1998. Dephosphorylation and deactivation of Ca^{2+}/calmodulin-dependent protein kinase II in BTC3-cells is mediated by Mg^{2+}- and okadaic-acid-sensitive protein phosphatases. Biochem. J. 329, 283–288.

Eppig, J., 1999. Mouse oocyte maturation, fertilization and preimplantation development in vitro. In: Richter, J. (Ed.), A Comparative Methods Approach to the Study of Oocytes and Embryos. Oxford Univ. Press, New York, pp. 3–9.

Fissore, R., Smyth, J., Kurokawa, M., Collas, P., 2002. Activation of mammalian oocytes. In: Cibelli, J., Lanza, R., Campbell, K., West, M. (Eds.), Principles of Cloning. Academic Press, San Diego, pp. 21–45.

Gordo, A., Wu, H., He, C., Fissore, R., 2000. Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of $[Ca^{2+}]_i$ oscillations and oocyte age. Biol. Reprod. 62, 1370–1379.

Hatch, K., Capco, D., 2001. Colocalization of CaMKII and MAP kinase on architectural elements of the mouse egg: potentiation of MAP kinase activity by CaMKII. Mol. Reprod. Dev. 58, 69–77.

Hogan, B., Costantini, F., Lacy, E., 1986. Manipulating the Mouse Embryo: a Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hudmon, A., Schulman, H., 2002. Structure-function of the multifunctional Ca^{2+}/Calmodulin-dependent protein kinase II. Biochem. J. 364, 593–611.

Huneau, D., Toth, S., Ozil, J.-P., 2003. Oocyte activation is the result of calcium signal summation. Reprod. Fertil. Dev. (Proceedings of International Embryo Transfer Society) 16, 269.

Ishida, A., Kameshita, I., Okuno, S., Kitani, T., Fujiwara, H., 1995. A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. Biochem. Biophys. Res. Commun. 212, 806–812.

Johnson, J., Bierle, B., Gallicano, G., Capco, D., 1998. Calcium/calmodulin dependent protein kinase II and calmodulin: regulators of the meiotic spindle in mouse eggs. Dev. Biol. 204, 464–477.

Jones, K., 1998. Ca^{2+} oscillations in the activation of the egg and development of the embryo in mammals. Int. J. Dev. Biol. 42, 1–10.

Jones, H., Lenz, R., Palevitz, B., Cormier, M., 1980. Calmodulin localization in mammalian spermatozoa. Proc. Natl. Acad. Sci. U. S. A. 77, 2772–2776.

Jones, K., Carroll, J., Whittingham, D., 1995. Ionomycin, thapsigargin, ryanodine, and sperm induced Ca^{2+} release increase during meiotic maturation of mouse oocytes. J. Biol. Chem. 270, 6671–6677.

Kim, S., Hudmon, A., Volmer, A., Waxham, M., 2001. CaM-Kinase II dephosphorylates Thr^{286} by a reversal of the autophosphorylation reaction. Biochem. Biophys. Res. Commun. 282, 733–780.

Kishikawa, H., Wakayama, T., Yanagimachi, R., 1999. Comparison of oocyte-activating agents for mouse cloning. Cloning 1, 153–159.

Kurokawa, M., Fissore, R., 2003. ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. Mol. Hum. Reprod. 9, 523–533.

Lawrence, Y., Ozil, J.-P., Swann, K., 1998. The effects of 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.

Lorca, T., Cruzalegul, F., Fesquet, D., Cavadore, J.-C., Méry, J., Means, A., Dorée, M., 1993. Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. Nature 366, 270–273.

Lorca, T., Castro, A., Martinez, A., Vigneron, S., Morin, N., Sigrist, S., Lehner, C., Doree, M., Labbe, J., 1998. Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts. EMBO J. 17, 3565–3575.

Markoulaki, S., Matson, S., Abbott, A., Ducibella, T., 2003. Oscillatory CaMKII activity in mouse egg activation. Dev. Biol. 258, 464–474.

Mehlmann, L., Kline, D., 1994. Regulation of intracellular calcium in the mouse egg: calcium release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. Biol. Reprod. 51, 1088–1098.

Menegon, A., Verderio, C., Leoni, C., Benfenati, F., Czernik, A., Green-

gard, P., Matteoli, M., Valtorta, F., 2002. Spatial and temporal regulation of Ca^{2+}/calmodulin-dependent protein kinase II activity in developing neurons. J. Neurosci. 22, 7016–7026.

Miyazaki, S., Shirakawa, H., Nakada, K., Honda, Y., 1993. Essential role of inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. Dev. Biol. 158, 62–78.

Moos, J., Kopf, G., Schultz, R., 1996. Cycloheximide-induced activation of mouse eggs: effects of cdc2/cyclin B and MAP kinase activities. J. Cell Sci. 109, 739–748.

Nixon, V., Levasseur, M., McDougall, A., Jones, K., 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., 1990. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. Development 109, 117–127.

Ozil, J.-P., 1998. Role of calcium oscillations in mammalian egg activation: experimental approach. Biophys. Chem. 72, 141–152.

Ozil, J.-P., Huneau, D., 2001. Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development. Development 128, 917–928.

Reimann, J., Jackson, P., 2002. Emi1 is required for cytosolic factor arrest in vertebrate eggs. Nature 416, 850–854.

Runft, L., Jaffe, L., Mehlmann, L., 2002. Egg activation at fertilization: where it all begins. Dev. Biol. 245, 237–254.

Saunders, C., Larman, M., Parrington, J., Cox, L., Royse, J., Blayney, L., Swann, K., Lai, F., 2002. PLCζ: a sperm-specific trigger if Ca^{2+} oscillations in eggs and embryo development. Development 129, 3533–3544.

Schultz, R., Kopf, G., 1995. Molecular basis of mammalian egg activation. Curr. Top. Dev. Biol. 30, 21–62.

Shirayama, M., Toth, A., Galova, M., Nasmyth, K., 1999. APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. Nature 402, 203–207.

Strack, S., Barban, M., Wadzinski, B., Colbran, R., 1997. Differential inactivation of postsynaptic density-associated and soluble Ca^{2+}/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. J. Neurochem. 68, 2119–2128.

Stricker, S., 1999. Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev. Biol. 211, 157–176.

Swann, K., 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development 110, 1295–1302.

Swann, K., Ozil, J.-P., 1994. Dynamics of the calcium signal that triggers mammalian egg activation. Int. Rev. Cytol. 152, 183–222.

Tatone, C., Iorio, R., Francione, A., Gioia, I., Colonna, R., 1999. Biochemical and biological effects of KN-93, an inhibitor of calmodulin-dependent protein kinase II, on the initial events of mouse egg activation induced by ethanol. J. Reprod. Fertil. 115, 151–157.

Tatone, C., Delle Monache, S., Iorio, R., Caserta, D., Di Cola, M., Colonna, R., 2002. Possible role for Ca^{2+} calmodulin-dependent protein kinase II as an effector of the fertilization Ca^{2+} signal in mouse oocyte activation. Mol. Hum. Reprod. 8, 750–757.

Vitullo, A., Ozil, J., 1992. Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. Dev. Biol. 151, 128–136.

Winston, N., Maro, B., 1995. Calmodulin-dependent protein kinase II is activated transiently in ethanol-stimulated mouse oocytes. Dev. Biol. 170, 350–352.

Winston, N., Maro, B., 1999. Changes in the activity of type 2A protein phosphatases during the meiotic maturation and the first mitotic cell cycle in mouse oocytes. Biol. Cell 91, 175–183.

Wu, J., Ribar, T., Means, R., 2001. Spermatogenesis and the regulation of Ca^{2+}-calmodulin-dependent protein kinase IV localization are not dependent of calspermin. Mol. Cell. Biol. 21, 6066–6070.

Xu, Z., Lefevre, L., Ducibella, T., Schultz, R., Kopf, G., 1996. Effects of calcium-BAPTA buffers and the calmodulin antagonist W-7 on mouse egg activation. Dev. Biol. 180, 594–604.