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Egg activation events are regulated by the duration of a sustained $[Ca^{2+}]_{cyt}$ signal in the mouse

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Abstract

Although the dynamics of oscillations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) play important roles in early mammalian development, the impact of the duration when $[Ca^{2+}]_{cyt}$ is elevated is not known. To determine the sensitivity of fertilization-associated responses [i.e., cortical granule exocytosis, resumption of the cell cycle, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity, recruitment of maternal mRNAs] and developmental competence of the parthenotes to the duration of a $[Ca^{2+}]_{cyt}$ transient, unfertilized mouse eggs were subjected to a prolonged $[Ca^{2+}]_{cyt}$ change for 15, 25, or 50 min by means of repetitive Ca^{2+} electropermeabilization at 2-min intervals. The initiation and completion of fertilization-associated responses are correlated with the duration of time in which the $[Ca^{2+}]_{cyt}$ is elevated, with the exception that autonomous CaMKII activity is down-regulated with prolonged elevated $[Ca^{2+}]_{cyt}$. Activated eggs from 25- or 50-min treatments readily develop to the blastocyst stage with no sign of apoptosis or necrosis and some implant. Ca^{2+} influx into unfertilized eggs causes neither Ca^{2+} release from intracellular stores nor rapid removal of cytosolic Ca^{2+}. Thus, the total Ca^{2+} signal input appears to be an important regulatory parameter that ensures completion of fertilization-associated events and oocytes have a surprising degree of tolerance for a prolonged change in $[Ca^{2+}]_{cyt}$.

Keywords: Egg activation; Electropermeabilization; Ca^{2+} signal duration; CaMKII; Phosphoinositide; Preimplantation mouse embryo

Introduction

Considerable progress has been made in understanding how mammalian sperm induces a series of intracellular calcium oscillations ($[Ca^{2+}]_{cyt}$) that are essential for oocyte activation and embryo development (Kouchi et al., 2004; Saunders et al., 2002; Swann et al., 2004). Injection of a novel sperm-specific phospholipase C (PLCζ) into mouse unfertilized eggs triggers an initial $[Ca^{2+}]_{cyt}$ transient followed by a series of smaller $[Ca^{2+}]_{cyt}$ spikes, resembling those observed after fertilization (Kouchi et al., 2004; Kurokawa et al., 2004; Saunders et al., 2002). Moreover, with experimental down-regulation of PLCζ expression in sperm, fertilized eggs have an absence or decrease of normal oscillations and fail to develop to term (Knott et al., 2005). These studies clearly demonstrate that the frequency of $[Ca^{2+}]_{cyt}$ oscillations varies in relation to the concentration of PLCζ injected into the egg (Swann et al., 2004). However, despite the increasing knowledge in signal transduction mechanisms, the functional linkage between frequency, number, amplitude, and duration of the Ca^{2+} signal and development is not established (Kurokawa and Fissore, 2003).

Oscillations in $[Ca^{2+}]_{cyt}$ in somatic cells led to hypotheses of frequency modulation coding (FM mode) or amplitude modulation coding (AM mode) (Berridge

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and Galione, 1988; Dolmetsch et al., 1997; Rapp et al., 1981). In mammalian fertilization, however, the question of AM versus FM mode remains elusive because eggs can respond to both modes. A single but large monotonic $[Ca^{2+}]_{cyt}$ increase can activate the egg and promote parthenogenetic preimplantation development (Cuthbertson, 1983; Surani et al., 1984). Alternatively, the number of Ca^{2+} oscillations can regulate early events of egg activation, (Ducibella et al., 2002; Kline and Kline, 1992; Lawrence et al., 1998) as well as pre- and post-implantation development of parthenotes (Bos-Mikich et al., 1997; Ozil and Huneau, 2001). Moreover, because normal offspring can be obtained after introducing a male pronucleus taken from a fertilized egg into an egg that has been artificially activated by either alcohol treatment (Surani et al., 1984) or repetitive electrical stimulation (Ducibella et al., 2002), mammalian eggs appear capable of correctly responding to both AM and FM modes of Ca^{2+} signaling.

Therefore, the question arises whether the repetitive pattern of Ca^{2+} oscillations simply reflects the intrinsic regulative properties of the Ca^{2+} signaling toolkit (Berridge et al., 2003)—mainly the kinetic properties of phosphoinositide hydrolysis, production of $InsP_3$, and $InsP_3$ receptor and intraluminal Ca^{2+} fluxes. Or, alternatively, oscillations are an absolute prerequisite for regulating and optimizing early events that in turn are coupled to later developmental responses (Ducibella et al., 2002; Ozil and Huneau, 2001). The first does not imply the existence of a specific Ca^{2+} signal decoder, whereas the second implies the existence of either a spike counter (Meyer and Stryer, 1991) or a frequency decoder (Dupont et al., 1991; Goldbeter et al., 1990).

To ascertain whether an oscillatory Ca^{2+} signal is necessary, we sustained experimentally the duration of a $[Ca^{2+}]_{cyt}$ increase beyond its physiological limits using a method described previously (Ozil and Swann, 1995). We show here that completion of meiosis and fertilization-associated events does not require fine-tuning of the dynamics of Ca^{2+} signaling. In addition, early cellular and molecular events display a graded response to the duration of a single Ca^{2+} signal. The activated eggs readily develop to the blastocyst stage, with no sign of apoptosis or necrosis, and some of them implant. Therefore, the total Ca^{2+} signal input (TCSI) appears to be an important controlling parameter during egg activation. Nevertheless, the developmental competence of parthenotes activated by prolonged $[Ca^{2+}]_{cyt}$ is statistically lower than those derived from eggs subjected to repetitive Ca^{2+} signaling.

Materials and methods

Egg collection and embryo culture

Metaphase II (MII) eggs were obtained from the oviducts of F1 (C57BL/6 × CBA) female mice (6–8 weeks old) that were superovulated by i.p. injection of 5 IU of PMSG (Folligon® Intervet, Angers, France) followed 48 h later by injection of 7.5 IU of HCG (Chorulon® Intervet, Angers, France) to induce ovulation. Eggs were recovered in HEPES-buffered M2 medium (Sigma M7167) and cumulus cells were removed with 0.1% hyaluronidase (Sigma H-3506). Eggs were processed for Ca^{2+} stimulation 14–15 h after HCG administration. Fertilized eggs were obtained from superovulated F1 females mated to F1 males.

Embryo culture, activation treatments, and Ca^{2+} monitoring were all performed in M16 medium supplemented with 4 mg ml^{-1} of bovine serum albumin (Sigma A-3311) and equilibrated with 5% CO_2. Eggs studied for their developmental competence in vitro or in vivo were treated and cultured in the presence of 0.2 μg ml^{-1} of cytochalasin D (Sigma C-8273) for a total period of 6 h. Thus, the activated eggs did not form the second polar body and retained two sets of sister chromatids.

Measurement of intracellular free Ca^{2+} concentration

Eggs were incubated for 15 min at 37°C with 20 μM of the Ca^{2+}-sensitive probe Fura-2 AM (Molecular Probes F-1221). The dye was first diluted in DMSO containing Pluronic F-127 (final concentration 0.08% v/v and 0.016% w/v, respectively) and then diluted in M16 medium. The $[Ca^{2+}]_{cyt}$ was monitored by measuring the fluorescence from individual eggs loaded into a chamber (see below) placed on the stage of an inverted microscope (TE 2000 Nikon). Light excitation at 340 ± 5 nm and 380 ± 5 nm was selected from a Cairn monochromator and the fluorescence image acquisition was collected with a Photonic Science amplified ISIS CCD camera (Robertsbridge, UK). The free $[Ca^{2+}]_{cyt}$ is expressed as the 340/380 ratio of fluorescence (Imaging Workbench 2–4 Software, Axon Instruments). The resting level of intracellular Ca^{2+} was evaluated by washing an egg in a Ca^{2+}-free M16 and then subjecting the egg to an EF pulse of 100 V and 2 ms duration that irreversibly breached the plasma membrane. Once the membrane was breached, the egg was equilibrated for 1 min in Ca^{2+}-free medium, which makes it possible to measure the fluorescence ratio at low Ca^{2+} while a subsequent rapid perfusion with M16 containing 1.7 mM Ca^{2+} permitted measuring the ratio at saturating levels.

Calcium stimulation procedures for egg activation

Calcium ion influx was induced by transient membrane electropermeabilization in a special processor that assures rapid washing before the electrical pulse with a nonconductive medium (60 g l^{-1}; BDH Aristar, UK) of 100 μM Ca^{2+} in water (DirectQ™; Millipore, 18 MΩ cm) and very rapid washing after the pulse with culture medium to preserve cell viability as previously described (Ducibella et al., 2002; Ozil and Huneau, 2001; Ozil and Swann, 1995). When Ca^{2+} release from intracellular stores was required,

membrane electropermeabilization was performed in the presence of 20 μM $InsP_3$ (Sigma I-9766) (Ozil and Swann, 1995). Two slightly different chambers were designed to record $[Ca^{2+}]_{cyt}$ in individual eggs during electropermeabilization or activate batches of eggs (up to 100 eggs). During the experiment, the chamber temperature was maintained at 37°C. The electric field (EF) pulse parameter was composed of an oscillating electric field of 10 kHz during 300 μs.

Four permeabilization treatments were used. The first one, consisting of 24 pulses of 57 V amplitude (field strength: 1.42 kV cm^{-1}) given every 8 min for 3 h (named 24P-8 min), was used for injecting Ca^{2+} or Ca^{2+} and $InsP_3$. This caused a short duration $[Ca^{2+}]_{cyt}$ change every 8 min and served as a reference because this pattern of Ca^{2+} signaling initiates and drives events of fertilization to completion (Ducibella et al., 2002). Three other treatments were designed to cause a single $[Ca^{2+}]_{cyt}$ rise whose duration was sustained by the repetition of electrical pulses every 2 min to prevent restoration of the resting level of $[Ca^{2+}]_{cyt}$. The duration of the sustained $[Ca^{2+}]_{cyt}$ change was driven by the number of electrical pulses (3, 10, and 20), named hereafter 3P-2 min, 10P-2 min, and 20P-2 min, respectively. The voltage amplitude for these three treatments was increased to 65 V (field strength: 1.62 kV cm^{-1}) in order to maximize the transmembrane Ca^{2+} influx. When required, the value of the decay time after a $[Ca^{2+}]_{cyt}$ increase was measured as the time required for the $[Ca^{2+}]_{cyt}$ to decrease from 90% of the $[Ca^{2+}]_{cyt}$ amplitude to 10%.

Graphical estimate of the total Ca^{2+} signal input (TCSI)

In order to estimate the degree of Ca^{2+} stimulation, the total calcium signal input (TCSI) of a given treatment was estimated by summing the ratio data points for a given record. We hypothesized that the TCSI is primarily related to three parameters: (i) the quantity of extracellular Ca^{2+} that has moved into the cytosol; (ii) the time that these ions remain in the cell during each signal, and (iii) the number of Ca^{2+} stimulations. The quantitative interpretation in terms of average $[Ca^{2+}]_{cyt}$ is difficult to obtain because it is subject to sensitivity parameters of the imaging instrument. To address this issue, we analyzed records that have similar $[Ca^{2+}]_{cyt}$ amplitude. In addition, because unfertilized mouse eggs are not capable of spontaneously releasing Ca^{2+} from intracellular stores, we assume that the TCSI provides a reasonable estimate of the total duration during which the eggs are exposed to a similar high $[Ca^{2+}]_{cyt}$ amplitude. TCSI was generated by summing the fluorescence ratio (340/380) over time for four different but typical records. The baseline of the ratio before the first Ca^{2+} stimulation was subtracted from the entire range of data points to avoid accumulating values of the resting level for each data point. The Sigma Plot SUM function was then implemented to generate a new range of numbers representing the accumulated sums along the list that gives an estimate of the Ca^{2+} signal input "seen" by the egg during the time course of the treatment.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed with mouse sperm heads that were separated from their tails by applying a 0.1-s ultrasonic pulse from a digital sonicator (DS 250 Branson Ultrasonic Corporation, USA). The sperm head was injected into a freshly ovulated egg loaded with Fura-2 AM (see above) (Kimura and Yanagimachi, 1995; Kurokawa and Fissore, 2003), using a piezo micropipette-driving unit (Piezodrill Burleigh, USA). ICSI was performed in M2 medium at room temperature. Immediately after ICSI, the eggs were put on the imaging microscope equipped with the perfusion chamber for $[Ca^{2+}]_{cyt}$ monitoring.

Kinetics of egg activation and pronucleus formation

Eggs subjected to 24P-8 min, 3P-2 min, 10P-2 min, and 20P-2 min treatments in the presence of Ca^{2+} were cultured and observed with a Nikon SMZ1500 stereomicroscope for pronucleus (PN) formation at 1-h intervals from 1 to 9 h after the onset of treatment.

Immunofluorescence of cortical granules and chromatin

Cortical granule (CG) exocytosis and cell cycle stage were evaluated as previously described (Abbott et al., 1999; Xu et al., 1997).

CaMKII, histone H1, and MAP kinase activity assays

Autonomous Ca^{2+}/calmodulin(CaM)-dependent protein kinase II (CaMKII) activity, in the absence of any exogenous Ca^{2+}/CaM, as well as maximal activity (with excess Ca^{2+}/CaM), was assayed as previously described (Markoulaki et al., 2003). Unless otherwise indicated, CaMKII activity refers to autonomous activity, which reflects the relative extent of endogenously autophosphorylated CaMKII. Histone H1 kinase (H1K) activity (reflecting $p34^{cdc2}$/cyclin B kinase activity) and MAPK activities were each measured in single eggs as previously described (Moos et al., 1995; Xu et al., 1997).

[^{35}S]-Methionine metabolic radiolabeling and two-dimensional electrophoresis

As described previously (Xu et al., 1997), eggs were radiolabeled in medium containing 1 mCi/ml of [^{35}S]-methionine (specific activity 1000 Ci/mmol) for 6 h. The samples were then processed and subjected to two-dimensional (2-D) gel electrophoresis using the Investigator 2-D Electrophoresis System (Millipore). Equal numbers of acid insoluble counts were loaded and

subjected to 2-D gel electrophoresis, and radiolabeled proteins were detected by using a phosphorimager.

In vitro and in vivo development

To assess the in vitro developmental capacity, diploid embryos were cultured for 5 days and embryos that reached the blastocyst stage were scored. Two-cell stage diploid embryos were transferred into pseudopregnant F1 foster mothers, which were autopsied at day 8 of gestation to count the number of implantation sites and embryos.

Statistical analysis

For treated and control groups, data were expressed as the mean value per group and compared statistically

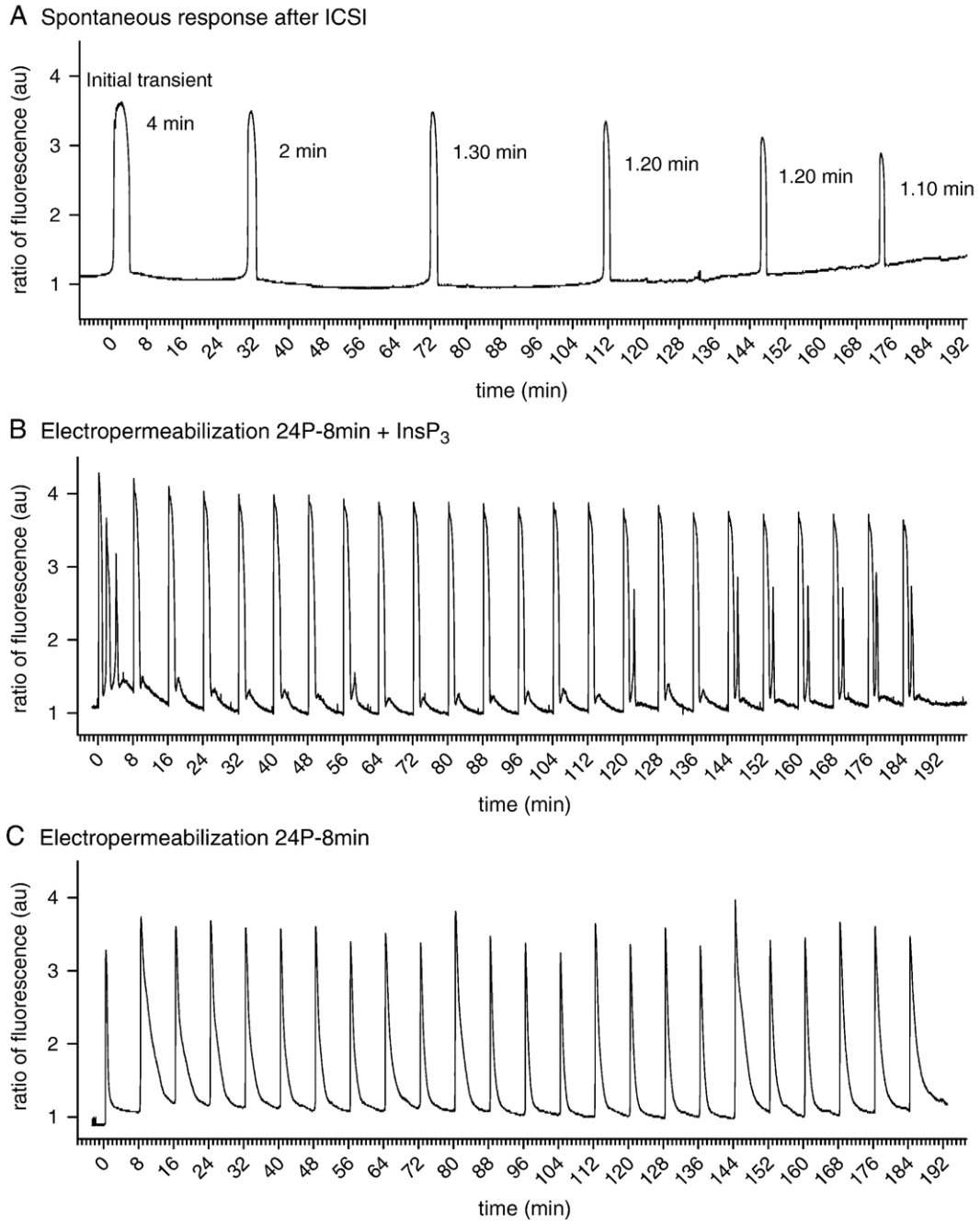

Fig. 1. $[Ca^{2+}]_{cyt}$ responses to ICSI or electropermeabilization. (A) ICSI. The initial longer duration $[Ca^{2+}]_{cyt}$ signal begins a few minutes after injection and is followed by a series of shorter $[Ca^{2+}]_{cyt}$ signals. Note that the duration of these $[Ca^{2+}]_{cyt}$ transients is progressively reduced over time. (B). Electropermeabilization. 24P-8 min treatment (57 V) in the presence of Ca^{2+} and 20 μM $InsP_3$. Every pulse causes a rapid increase followed by a rapid decrease, sometimes followed by spontaneous $[Ca^{2+}]_{cyt}$ oscillations. Note that the $[Ca^{2+}]_{cyt}$ profile does not change with the time. (C) Electropermeabilization. 24P-8 min treatment (57 V) in the presence of Ca^{2+}. Every pulse causes a rapid increase followed by a slow decrease, resulting in a consistent pattern.

with the χ^2 test, t test, or Mann–Whitney Rank Sum test.

Results

Experimental control of the Ca^{2+} signal pattern and duration

Spontaneous Ca^{2+} response after fertilization by ICSI

ICSI was used as a reference for the normal Ca^{2+} response. Every spontaneous signal, including the first (Figs. 1A and 2A,B), began with a slow rise followed by a rapid acceleration of Ca^{2+} release from intracellular stores that is typical of the Calcium Induced Calcium Release (CICR) process (Igusa and Miyazaki, 1983). The $[Ca^{2+}]_{cyt}$ signal was always terminated by a rapid removal of Ca^{2+} ions with an average decay time of 33.7 s ± 4.5 (n = 6, Figs. 1A and 2A,B, Table 1).

Membrane electropermeabilization

We first validated our experimental system with respect to the functional integrity of the eggs subjected to a series of Ca^{2+} influxes. The method of Ozil and Swann (1995) was used to preserve the functional integrity of the plasma membrane subjected to high voltage pulses. This method allows experimental manipulation of $[Ca^{2+}]_{cyt}$ and minimizes the side effects often associated with membrane electroporation (Swezey and Epel, 1989).

The pattern of $[Ca^{2+}]_{cyt}$ in an unfertilized egg subjected to the 24P-8 min treatment in the presence of Ca^{2+} or Ca^{2+} plus $InsP_3$ showed that each electrical pulse (57 V, every 8 min for 3 h) resulted in a similar $[Ca^{2+}]_{cyt}$ response (Figs. 1B,C). The $[Ca^{2+}]_{cyt}$ amplitudes were similar to the spontaneous Ca^{2+} signals seen after ICSI (Fig. 1A). Membrane properties after Ca^{2+} influx were preserved because no progressive increase in resting $[Ca^{2+}]_{cyt}$ or desensitization of the egg's response over time was observed. Unfertilized eggs subjected to these treatments formed blastocysts and were implanted (below). The experiments described below explore how the time of duration of the $[Ca^{2+}]_{cyt}$ signal can be experimentally manipulated in fertilized and unfertilized eggs.

Table 1
Decay time in an increase in $[Ca^{2+}]_{cyt}$

Treatment	Type of egg	Record	# Signals	Decay time in seconds ± SEM
None	ICSI	Fig. 1A	6	33.7 ± 4.5[b,c]
24P-8 min (Ca^{2+} + $InsP_3$)	Unfertilized	Fig. 1B	24	42.1 ± 1.4[b]
24P-8 min (Ca^{2+})	Unfertilized	Fig. 1C	24	140.1 ± 11.6[a]
20P-2 min (Ca^{2+})	ICSI	Fig. 3A	20	51.9 ± 2.2[d]

Statistical significance was calculated using the t test of the Mann–Whitney Rank Sum test. Groups with different superscript letters were significantly different ($P < 0.001$). 24P-8min = 24 pulses, 8 min frequency.

Ca^{2+} influx during ICSI causes Ca^{2+} release and uptake.

To reveal how fertilized eggs accommodate a series of Ca^{2+} influx in terms of signal duration, ICSI eggs were subjected to the 20P-2 min treatment with the highest voltage, i.e., 65 V. Every pulse causes a $[Ca^{2+}]_{cyt}$ signal that remained transiently elevated and then decreased (Figs. 3A,B, solid line), the pattern resembling the spontaneous pattern after ICSI. The duration of every signal was fairly constant (92.2 s ± 2.8) and similar to the duration of the spontaneous signal (Fig. 1A). The down stroke always reached the resting level before the next pulse, i.e., 2 min. The average decay time was of 51.9 s ± 2.2 (n = 20, Fig. 3A and Table 1). After cessation of the electrical pulsations, the eggs generated a series of spontaneous but dampened Ca^{2+} oscillations with shorter duration (47.5 ± 2.1). Animation of Fig. 3B solid line (in Appendix A) is available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1.

Ca^{2+} and $InsP_3$ influx in unfertilized eggs causes Ca^{2+} release and uptake.

In order to reproduce fertilization-like cytosolic increases and decreases in Ca^{2+} in unfertilized eggs

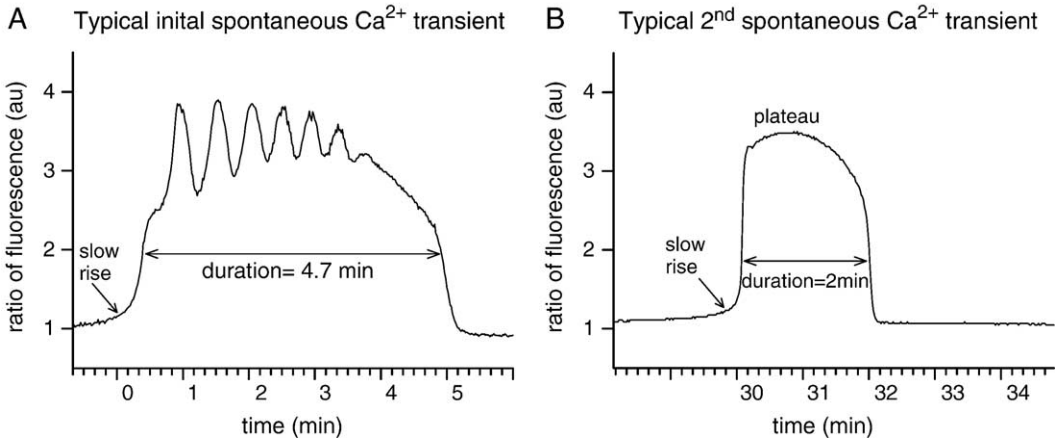

Fig. 2. $[Ca^{2+}]_{cyt}$ responses to ICSI. (A) A typical initial $[Ca^{2+}]_{cyt}$ transient is composed of a series of amplitude-damped oscillations before rapid Ca^{2+} reuptake occurs. The signal duration may vary between 3 and 8 min. (B) A typical second Ca^{2+} signal, which is always shorter than the first (as in panel A). The slow initial Ca^{2+} rise is followed by a rapid rate of increase, a plateau is reached and, finally, very efficient reuptake terminates the signal.

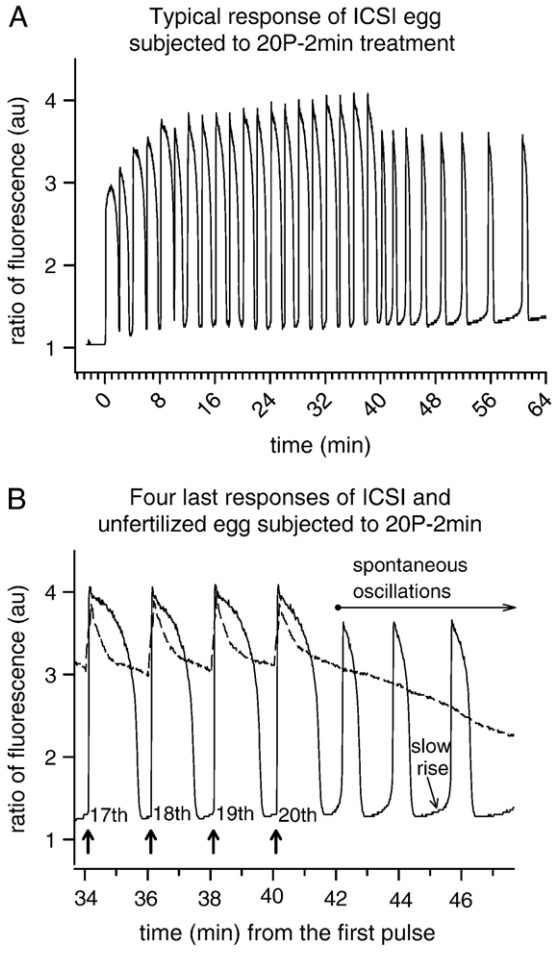

Fig. 3. $[Ca^{2+}]_{cyt}$ responses after ICSI and electropermeabilization. (A) With the 20P-2 min treatment, every EF pulse (65 V) causes a highly reproducible $[Ca^{2+}]_{cyt}$ increase and subsequent efficient Ca^{2+} reuptake, completed before the next EF pulse. After the 20th EF pulse, spontaneous $[Ca^{2+}]_{cyt}$ oscillations occur with a damped period. (B) Two records are superimposed. The solid line represents the 4 last responses of an ICSI egg subjected to 20P-2 min. Spontaneous oscillations are characterized by a slow rise after the cessation of the stimulation. The dashed line represents the 4 last responses of an unfertilized egg subjected to the same treatment. Note the absence of Ca^{2+} uptake between pulses and the slow decrease after cessation of the stimulation.

that are not sensitized to release Ca^{2+} from intracellular stores (Swann and Ozil, 1994), unfertilized eggs were subjected to EF pulses (24P-8 min) of low voltage, i.e., 57 V, in the presence of both Ca^{2+} and $InsP_3$. The pattern of the $[Ca^{2+}]_{cyt}$ signal was similar to that observed after ICSI (Fig. 1B versus A), i.e., a fast $[Ca^{2+}]_{cyt}$ increase at the moment of the electrical pulse followed by a short period of elevated $[Ca^{2+}]_{cyt}$ and a very rapid down stroke with an average decay time of 42.1 s ± 1.4 (n = 24, Figs. 1B and 4, solid line and Table 1). In some cases, a few typical $[Ca^{2+}]_{cyt}$ oscillations were observed after stimulation, revealing the existence of spontaneous Ca^{2+} release and uptake processes in the absence of fertilization (Fig. 1B). Nevertheless, it appears difficult to vary the duration of a single signal when it originates from CICR because the termination of the signal (Ca^{2+} uptake) appears self-regulated and coupled with the CICR process. Animation of Fig. 4 solid line (in Appendix A) is available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1.

Ca^{2+} influx in unfertilized eggs does not cause Ca^{2+} release and uptake. To understand better the functional link between Ca^{2+} release and Ca^{2+} uptake, unfertilized eggs were subjected to EF pulses (24P-8 min) of low voltage, i.e., 57 V, in the presence of Ca^{2+} alone. The record (Fig. 1C) shows a rapid $[Ca^{2+}]_{cyt}$ increase (Fig. 4, dashed line) that is not followed by a rapid recovery. The decay time is three times longer than the one observed after $InsP_3$ injection (decay time of 140.1 s ± 11.6, n = 24, Fig. 1C and Table 1). Hence, in addition to the fact that Ca^{2+} influx into unfertilized eggs is not capable of causing Ca^{2+} release from the ER, it is also incapable of causing a rapid cytosolic Ca^{2+} uptake. Therefore, as described next, we used this lack of rapid Ca^{2+} uptake in unfertilized egg to sustain elevated $[Ca^{2+}]_{cyt}$ by subjecting the eggs to a series of repetitive Ca^{2+} influxes. Animation of Fig. 4 dashed line (in Appendix A) is available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1.

Frequent Ca^{2+} influxes in unfertilized eggs cause sustained and elevated $[Ca^{2+}]_{cyt}$. We subjected unfertilized eggs to a higher voltage pulse (from 57 V to 65 V) at 2-min intervals. Due to the slow Ca^{2+} uptake, the Ca^{2+} profiles (Figs. 5A–C) revealed that these parameters resulted in a sustained high $[Ca^{2+}]_{cyt}$. The Ca^{2+} profile takes the form of a saw tooth pattern in which every electrical pulse causes a $[Ca^{2+}]_{cyt}$ peak and indicates that membrane integrity is not compromised. In these conditions, the total duration of the sustained $[Ca^{2+}]_{cyt}$ can be prolonged by increasing the number of pulses. The signal duration for the 3P-2 min, 10P-2 min and 20P-2 min treatment was approximately 15, 25, and 50 min, respectively, and terminated with a slow but

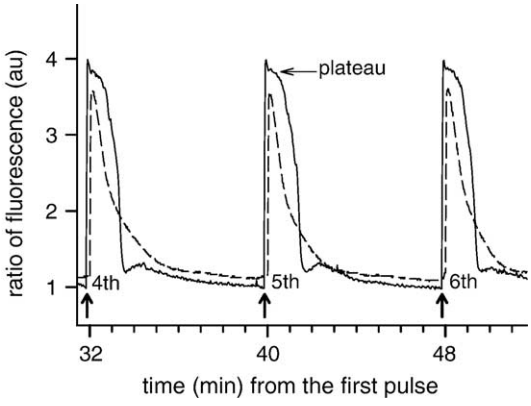

Fig. 4. $[Ca^{2+}]_{cyt}$ responses to three EF pulses in the presence of Ca^{2+} alone (dashed line), or with Ca^{2+} and 20 μM $InsP_3$ (solid line) from the fourth pulse of the 2 Ca^{2+} traces shown in Figs. 1B and C. The superposition of the two traces (EF pulses—arrows) shows the Ca^{2+} influx from the pulse, prolonged $[Ca^{2+}]_{cyt}$ release from stores by $InsP_3$, the absence of a slow rise before the pulse, and a relatively rapid Ca^{2+} uptake after Ca^{2+} and $InsP_3$.

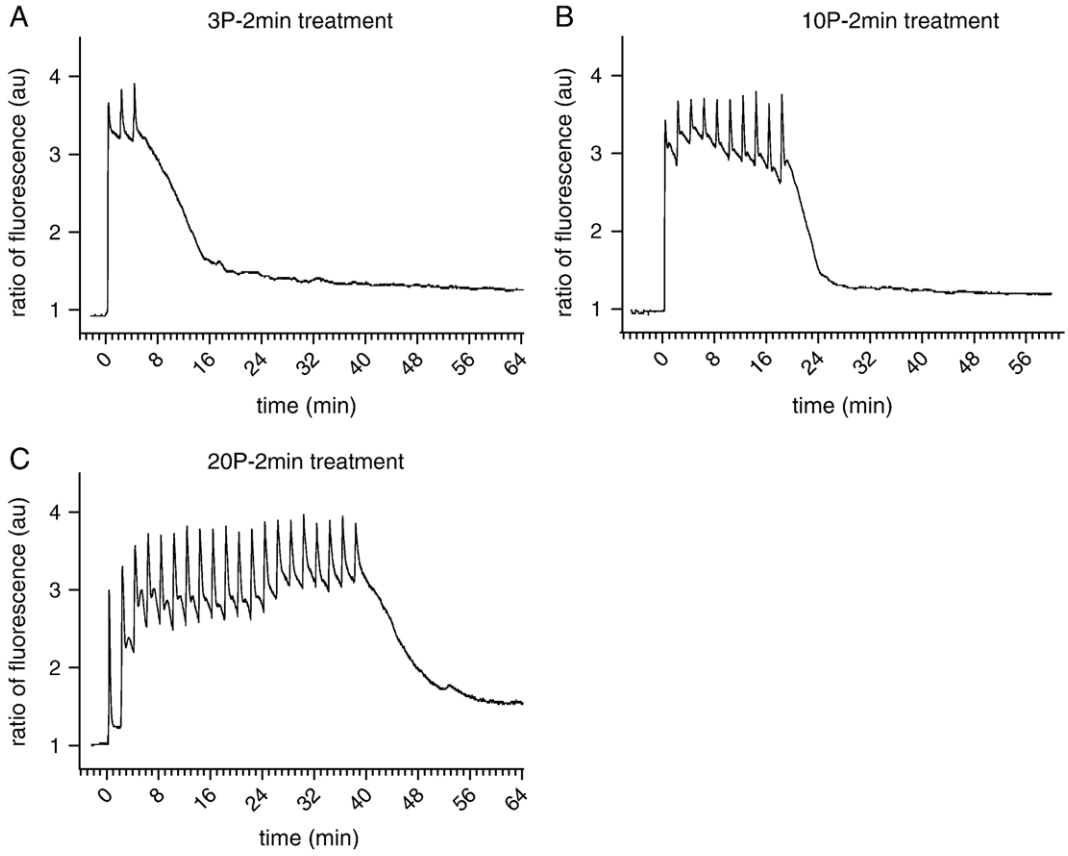

Fig. 5. Electropermeabilization with 3P-2 min, 10P-2 min, and 20P-2 min treatments (A, B, and C, respectively). Every EF pulse (65 V) causes a peak in $[Ca^{2+}]_{cyt}$. The $[Ca^{2+}]_{cyt}$ stays high between pulses and returns slowly to the resting level after cessation of EF pulses. The average duration of the signal is approximately 15, 25, and 50 min, respectively.

incomplete decrease. Animation of Fig. 5 (in Appendix A) is available at http://www.jcb.org/cgi/content/full/jcb.200311023/DC1.

Relative amplitude of $[Ca^{2+}]_{cyt}$ changes

The absolute level of the maximum $[Ca^{2+}]_{cyt}$ amplitude caused by the treatment was not measured. However, the relative amplitude of the fluorescence ratio (340 nm/380 nm) between low and saturating cytosolic level shown in Fig. 6 indicated that the maximum $[Ca^{2+}]_{cyt}$ level caused by the treatment was just below the saturating level of Fura-2 fluorescence (~1 μM; Grynkiewicz et al., 1985).

Graphical estimate of the total Ca^{2+} signal input (TCSI)

The graphical representation of the TCSI (see Materials and methods) delivered by the treatment integrates the amplitude of the $[Ca^{2+}]_{cyt}$ and the duration of the change (Fig. 7). Because the records taken for these plots have similar ratio amplitudes (Fig. 5), their respective TCSI gives a good representation of the time duration during which the eggs are exposed to similar $[Ca^{2+}]_{cyt}$ (see Materials and methods section). Hence, the 3P-2 min ($TCSI_{3P\text{-}2\ min}$) appeared similar to the one delivered by the first 8 pulses from the 24P-8 min ($TCSI_{8P\text{-}8\ min}$), the $TCSI_{10P\text{-}2\ min}$ was roughly equivalent to the $TCSI_{12P\text{-}8\ min}$, and the $TCSI_{20P\text{-}2\ min}$ to the $TCSI_{24P\text{-}8\ min}$. Such graphical representation of the TCSI made it possible to explore the

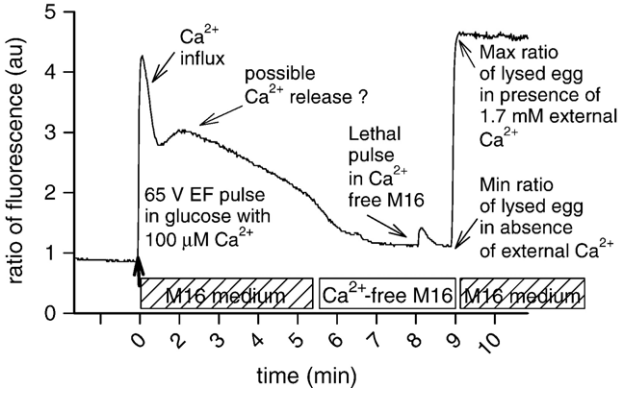

Fig. 6. Relative ratio fluorescence amplitude. Changes in $[Ca^{2+}]_{cyt}$ induced by an EF pulse of 65 V in the presence of 100 μM Ca^{2+} in low conductive medium. The large Ca^{2+} influx sometimes causes a Ca^{2+} rebound. After stimulation and washing in Ca^{2+}-free medium, a lethal pulse of 100 V (2 ms) in Ca^{2+}-free medium (arrow at 8 min) results in a transient small increase followed by a lower value that is a good estimate of the resting level of Ca^{2+}. At 9 min, the lethally permeabilized egg is washed with Ca^{2+} containing M16 medium and the fluorescence ratio provides a useful estimate of the saturation level for Fura-2.

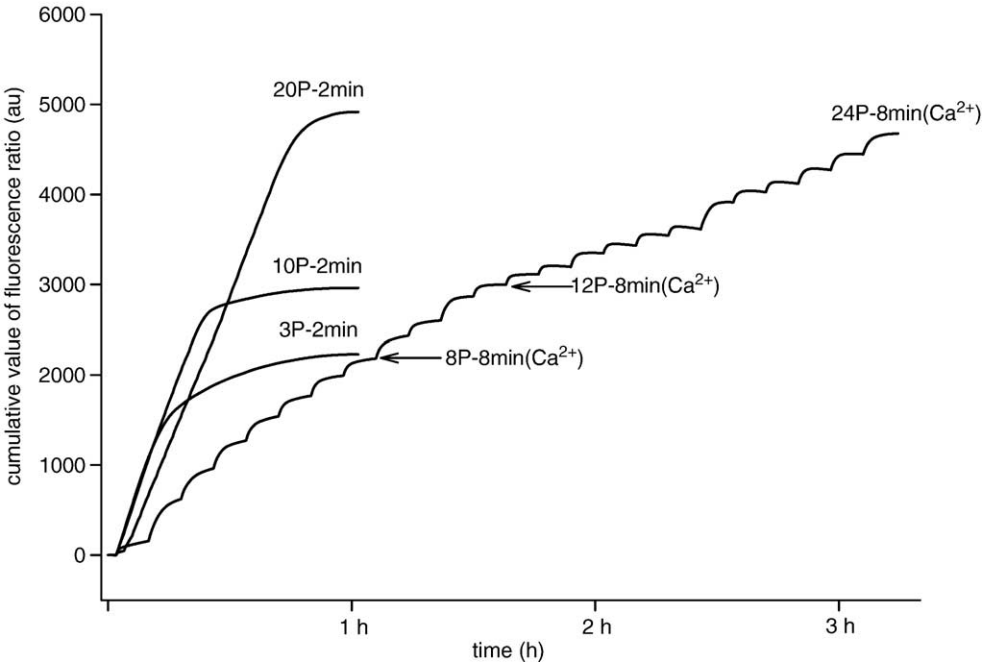

Fig. 7. Graphical representation of the total calcium signal input (TCSI, see text) from eggs with the different treatments indicated. Each curve represents the summation of the fluorescence ratio over time from a Ca^{2+} trace (Figs. 1C and 5). Note that the amplitude of the TCSI appears similar between the 20P-2 min and the 24P-8 min (Ca^{2+}). Moreover, the sharp Ca^{2+} peaks on the records in Fig. 5 are no longer apparent after TCSI summation, suggesting that their relative physiological impact is negligible.

relationship between the duration of the $[Ca^{2+}]_{cyt}$ changes during the time course of the treatment and the resulting biological responses.

Events of egg activation

We previously demonstrated that many events of egg activation are linked to the number of $[Ca^{2+}]_{cyt}$ signals delivered by the 24P-8 min (57 V) treatment (Ducibella et al., 2002). CG exocytosis was the most sensitive (initiated by 1–4 signals), cell cycle resumption required 4–8, and 24 were needed for entry into interphase. Recruitment of maternal mRNAs required 8 signals and became more apparent with 24 signals. The experiments below ascertained if a single $[Ca^{2+}]_{cyt}$ increase of varying total duration could substitute for a repetitive pattern stimulation, including whether 24 Ca^{2+} pulses could be replaced by a sustained exposure of elevated $[Ca^{2+}]_{cyt}$ with a similar total duration.

CG exocytosis

Each of the three sustained regimens resulted in a decrease in CG density of about 50% within 1 h (Fig. 8). At 2 h, there was additional CG loss in the 20P-2 min group that did not occur in the other two groups. Between 2 and 8 h, CG density in all three groups remained relatively constant. For the total time period (8 h), the percent CG loss in the 3P-2 min, 10P-2 min, and 20P-2 min groups was 47%, 58%, and 79%, respectively. The latter trend in CG loss with increasing Ca^{2+} exposure is similar to that previously reported for 4, 8, and 24 Ca^{2+} pulses with 8 min frequency (Ducibella et al., 2002).

Cell cycle progression

Cell cycle progression was monitored with chromatin staining and biochemically by measuring changes in MPF and MAP kinase activities. Progression of eggs through the cell cycle was assessed 8 h after the first EF pulse by DNA staining (Fig. 9). Untreated, control eggs were arrested at MII as expected. After 3 pulses at 2-min intervals, only 50% of eggs exited MII, but their chromatin did not undergo decondensation. These eggs were blocked either in anaphase II/telophase II of meiosis, or in "metaphase III" after having extruded the second polar body. MIII is characterized by condensed chromosomes and high MPF and MAP kinase activities, accounting for the failure of entry into interphase (Ducibella et al., 2002). After 10 pulses at 2-min interval, all eggs resumed meiosis, but only 12% of these formed PN, while the others were blocked in the intermediate stages (above). Twenty pulses given at 2-min intervals were necessary to drive eggs into interphase, as indicated by the formation of a PN (81%). Consistent with these results and those of our previous study (Ducibella et al., 2002), MPF activity underwent a transient decrease and MAP kinase activity was unaltered following the 10P-2 min treatment (Fig. 10). Only the 20P-2 min treatment resulted in a permanent decrease in the activities of both protein kinases.

Rate of egg activation

The percentage of eggs with PN is an excellent index of the completion of meiosis and entry into the first interphase of development (Figs. 9 and 11). The 24P-8 min (Ca^{2+} alone, or

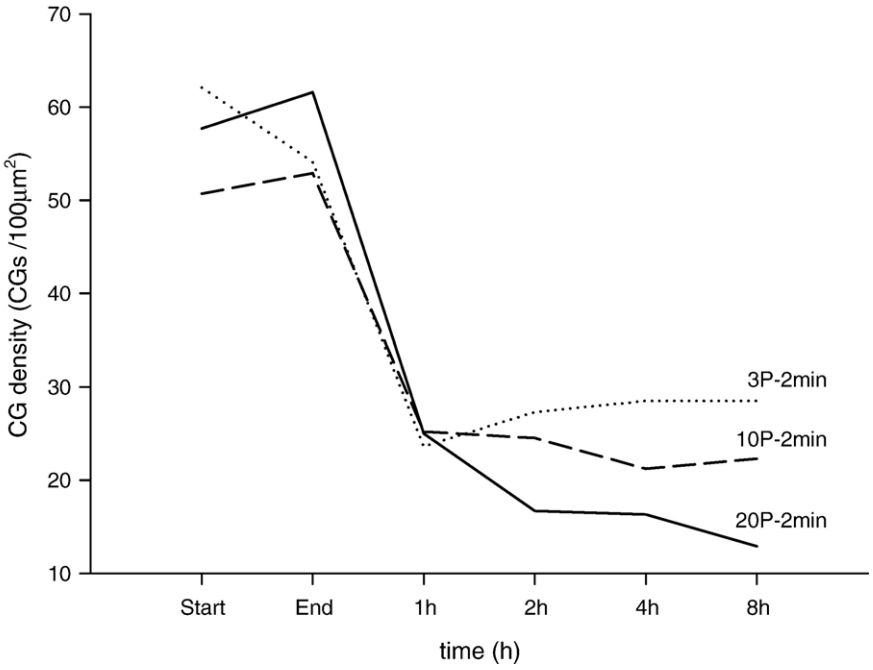

Fig. 8. Quantification of CG exocytosis in response to the three different pulse protocols. CG density is also provided for untreated "start" controls before treatment and untreated "end" controls after 8 h. In each experiment, more than 10 eggs were analyzed for each time point (controls; treated—1, 2, 4, 8 h). The 3, 10, and 20 pulses treatments were performed once, three times, and twice, respectively, and the mean values are shown.

Ca^{2+} and $InsP_3$) always gave a higher incidence of activation; 94% and 100%, respectively (Table 2). The 3P-2 min treatment, which maintained a high $[Ca^{2+}]_{cyt}$ for ~10 min, was unable to promote a high incidence of activation because only 5 out of 229 oocytes (2%; Table 2) formed a visible PN 6 h post-stimulation. The activation rate increased with the number of pulses and reached 38% (122/323) for the 10P-2 min and 81% (334/410) for the 20P-2 min (Table 2). In these cases, the earliest response was seen 4 h after stimulation and was essentially completed by 7 h with highest variability between replicates for the 10P-2 min treatment. This prolonged duration of the $[Ca^{2+}]_{cyt}$ signal did not cause

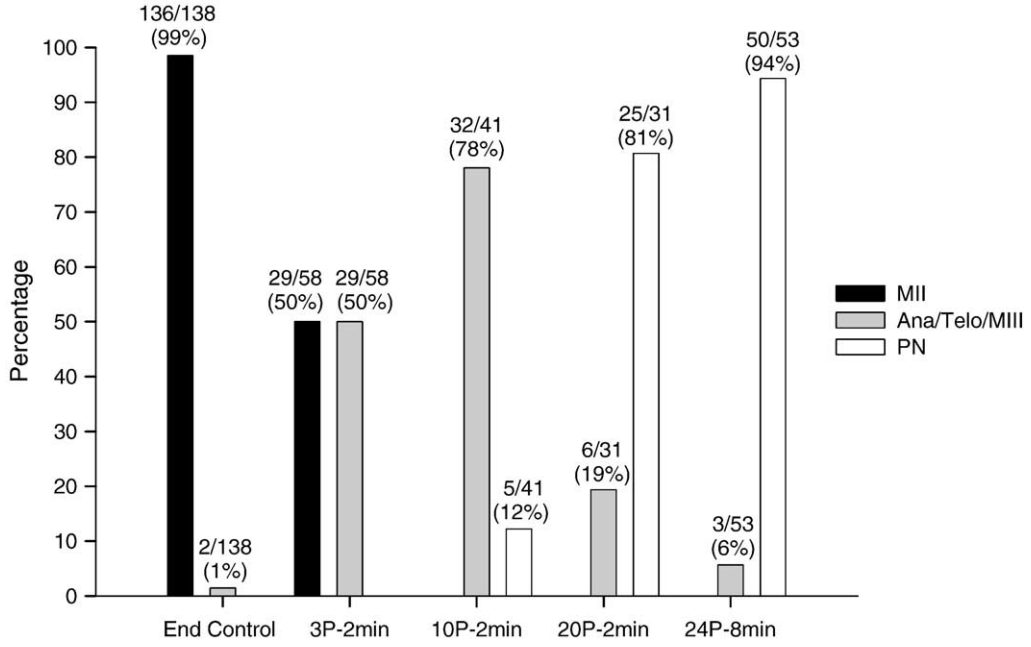

Fig. 9. Percentage of eggs in MII, MIII, and PN after the treatment. Eight hours after the onset of treatment, eggs were fixed for cell cycle analysis by DNA staining. Mean values are given for the % of eggs in a specific stage of the cell cycle. Each experiment (with a different pulse number) was repeated 2 or 3 times, and each time >12 eggs were analyzed. MII—metaphase II, Ana—anaphase, Telo—telophase, MIII—"metaphase III" (see Results), PN—pronucleus.

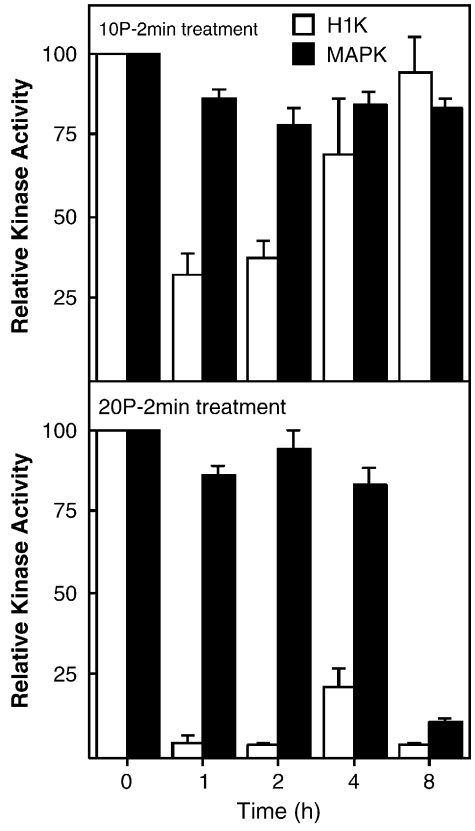

Fig. 10. Effect of the $[Ca^{2+}]_{cyt}$ signal duration treatment on protein kinase activities. The two panels (A and B) represent the relative % activity of H1K and MAPK for the treatment 10P-2 min and 20P-2 min, respectively. Note that 10P-2 min treatment causes a transient decrease only in H1K activity (A), whereas 20P-2 min (B) results in decreases in both kinase activities as observed after fertilization (see text). Each time point vertical bar represents the mean of 15 eggs (i.e., 3 separate experiments with 5 individually analyzed eggs per assay). Controls were from the onset of the experiment, prior to EF treatment. Bars represent SEM.

morphological abnormalities and normal late events of egg activation were observed—segregation of sister chromatids (above) and formation of two PN (Fig. 14A).

Recruitment of maternal mRNAs

The recruitment of maternal mRNAs, which results in changes in the pattern of protein synthesis, is the primary mechanism to express new proteins following egg activation (Schultz, 2002). The control (0P) and 3P-2 min groups had similar expression patterns (Fig. 12). In contrast, the 10P-2 min treatment resulted in the increased net synthesis of some polypeptides (Fig. 12C). Further apparent up-regulation was detected after 20P-2 min group (Fig. 9D) and this pattern was similar to that with 24P-8 min (Fig. 9E). The expression of spindlin isoforms, associated with the mouse egg meiotic spindle and whose mRNA is recruited following egg activation (Oh et al., 1997), changed with the duration of elevated $[Ca^{2+}]_{cyt.}$ (Fig. 12, box). The 24P-8 min pattern is similar to that after fertilization (Ducibella et al., 2002).

CaMKII activity in response to EF-induced Ca^{2+} rises

CaMKII activity, which is required for egg activation (Markoulaki et al., 2003; Tatone et al., 2002), is sensitive to the temporal aspects and amplitude of $[Ca^{2+}]_{cyt}$ signaling in eggs (Markoulaki et al., 2003, 2004). We examined the effect of increasing the duration of a single large $[Ca^{2+}]_{cyt}$ rise on CaMKII (autonomous) activity. After ~4 min of elevated $[Ca^{2+}]_{cyt}$, during the 2nd EF pulse, CaMKII activity was increased from 100% in untreated, control eggs to 234% (Fig. 13). Similarly, after ~10 min of elevated $[Ca^{2+}]_{cyt}$, during the 5th EF pulse, enzyme activity was increased to 210%.

However, after 20 and 40 min of elevated $[Ca^{2+}]_{cyt}$, during the 10th and 19th EF pulses, CaMKII activity had decreased to 120% and 87%, respectively. To investigate whether the enzyme became insensitive to stimulation by Ca^{2+}, untreated control eggs and eggs after ~20 min of elevated $[Ca^{2+}]_{cyt}$ were assayed for maximal CaMKII activity. Maximal activity (unlike autonomous activity) is a measure of total available enzyme activity under conditions of excess Ca^{2+} and CaM (see Materials and methods). The 20-min EF-treated eggs had a mean maximal CaMKII activity that was substantially lower (51%) compared to the maximal activity of untreated control eggs (100%). Thus, this duration of treatment appeared to reduce the Ca^{2+} sensitivity or amount of the enzyme. (Controls: $n = 9$; 20 min: $n = 8$; 3 experiments, 3 eggs/determination; t test $P = 0.015$).

Parthenogenetic development

There is a growing appreciation that the extent of parthenogenetic development postimplantation is coupled to the number of $[Ca^{2+}]_{cyt}$ oscillations during egg activation (Ozil and Huneau, 2001). The developmental potential of diploid parthenotes was evaluated by in vitro culture for 5 days or by transfer at the 2-cell stage of groups of ten embryos to pseudopregnant recipient mice that were autopsied at day 8 (D8) of gestation (Figs. 14A–D, Tables 3 and 4). Eggs subjected to the 24P-8 min (Ca^{2+}) treatment developed to the blastocyst stage (146/192, 76%; Table 3). Eggs subjected to 24P-8 min with Ca^{2+} alone or both Ca^{2+} and $InsP_3$ were transplanted at 2-cell stage and implanted at a rate of 72% (36/50) and 70% (99/141), respectively (Table 4). In contrast, although the incidence of blastocyst formation of eggs subjected to the 10P-2 min and 20P-2 min treatments was similar, 79% (49/62) and 75% (64/85), respectively, the incidence of implantation dropped to 45% (18/40) and 48% (32/67), respectively (Table 4). Regarding implantation sites after the 10P-2 min and 20P-2 min treatments, 2 fetuses and 1 fetus, respectively, were found (Figs. 14E–F, Table 4).

Discussion

In a previous study, we demonstrated that a series of fertilization events (e.g., CG exocytosis, cell cycle resumption with concomitant decreases in MPF and MAP kinase

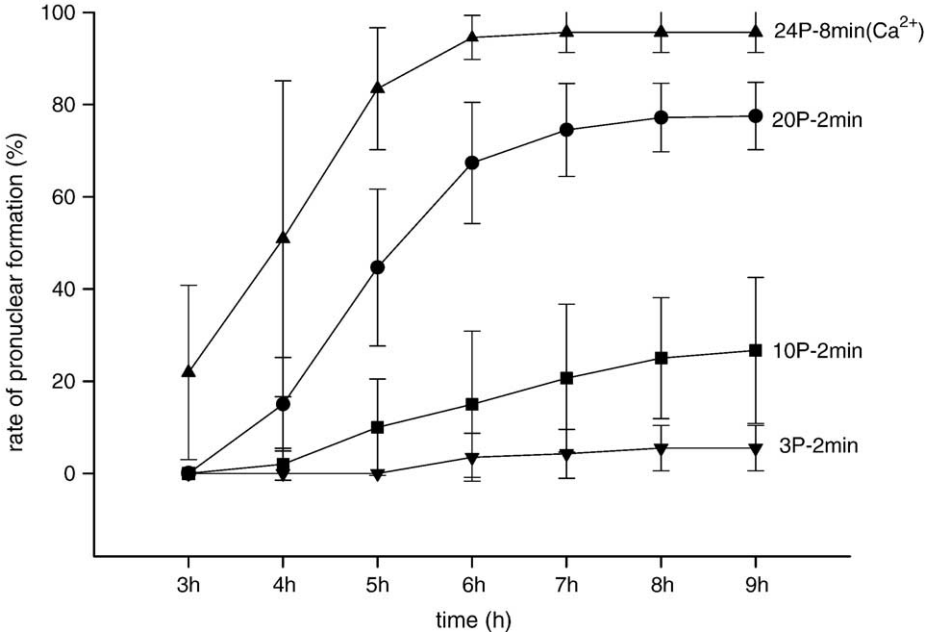

Fig. 11. Time course of pronucleus formation. Each symbol represents the mean value. Error bars represent the standard deviation.

activities, and recruitment of maternal mRNAs) are initiated and differentially regulated by the number of $[Ca^{2+}]_{cyt}$ oscillations (Ducibella et al., 2002). Here, we report that these fertilization events can also be initiated and regulated similarly by the duration of a single $[Ca^{2+}]_{cyt}$ increase with a fertilization-like amplitude. Interestingly, when the monotonic $[Ca^{2+}]_{cyt}$ signal duration is similar to that of the sum of the individual signal durations necessary for full egg activation, a high percentage of development to the blastocyst stage is observed. Nevertheless, the percentage of PN formation and, more importantly, the developmental potential of the blastocysts that results from a long monotonic $[Ca^{2+}]_{cyt}$ signal, are compromised in comparison to embryos that develop following fertilization-like oscillatory Ca^{2+} stimulation. In addition, efficient removal of Ca^{2+} from the cytosol was observed only when Ca^{2+} was released from internal stores. These biological and Ca^{2+} responses open a series of new questions regarding the functional role of the Ca^{2+} signal duration and its impact on the developmental processes.

Table 2
Extent of egg activation in response to Ca^{2+} treatments

Treatment	# Replicates	# Eggs	# Activated eggs recorded 8 h after the 1st pulse
24P-8 min (Ca^{2+})	9	419	395 (94%)[a]
24P-8 min (Ca^{2+} + $InsP_3$)	2	190	190 (100%)[b]
3P-2 min	9	229	5 (2%)[c]
10P-2 min	13	323	122 (38%)[d]
20P-2 min	15	410	334 (81%)[e]

Groups with different letters were significantly different (χ^2 $P \leq 0.002$).

The duration of sustained $[Ca^{2+}]_{cyt}$ regulates fertilization events and biological responses

The electropermeabilization method described here makes it possible to examine the relative dependencies of specific molecular and cellular events on exogenous Ca^{2+} influx in the absence of apparent phosphoinositide turnover and Ca^{2+} release from the ER (see below). The relative efficiency of treatments applied to an unfertilized egg can be compared because the signal input is related primarily to the quantity of extracellular Ca^{2+} ions that have moved into the cytosol where they continue to reside. It is important to note that some events of egg activation are sensitive to the amplitude of $[Ca^{2+}]_{cyt}$ (Nixon et al., 2002; Xu et al., 1996), and, thus, integrating the duration of time and $[Ca^{2+}]_{cyt}$ for different events is likely to be relevant. The TCSI delivered by 3P-2 min, 10P-2 min, and 20P-2 min highly correlates with the extent of egg activation and the kinetics of PN formation (Fig. 9). As with oscillatory Ca^{2+} signaling (Ducibella et al., 2002), many Ca^{2+}-dependant pathways involved in egg activation proceed in a graded (not threshold) manner when subjected to sustained elevated $[Ca^{2+}]_{cyt}$ signals of increasing duration. Nevertheless, our comparison of sustained versus repetitive Ca^{2+} stimulation reveals notable dosage and temporal differences. First, although significant % PN formation is stimulated by both methods, the final % PN after 8 h is slightly lower for 20P-2 min (81%, sustained Ca^{2+}) than from 24P-8 min (94% repetitive Ca^{2+}). Second, the 8P-8 min (repetitive) treatment (Ducibella et al., 2002) elicits greater CG exocytosis than the 3P-2 min (sustained) treatment despite a similar TCSI. Third, a treatment of about 1-h duration with high TCSI such as the 20P-2 min (sustained) gives a high rate of PN

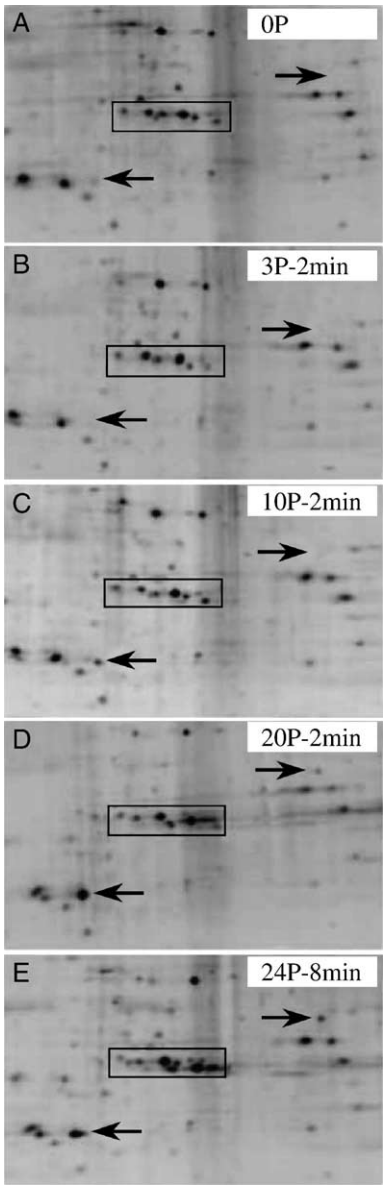

Fig. 12. Protein synthetic pattern is regulated by the $[Ca^{2+}]_{cyt}$ signal duration. Whole gels, each of which represents a different number of pulses, as indicated. In all groups, 4 h after the first Ca^{2+} stimulation, [^{35}S] methionine was added and the samples metabolically radiolabeled for 6 h. In response to an increasing duration of the monotonic $[Ca^{2+}]_{cyt}$ increase, note that the relative expression of many proteins appears to be up-regulated (horizontal arrows, rectangle), whereas some proteins are down-regulated. Changes are also observed in the pattern of the spindlin protein complex (rectangle) (see text). The experiment was conducted three times, and similar results were obtained each time. Shown are representative examples from one experiment. Twenty-five eggs were used per gel.

formation (81%; Table 2), whereas a repetitive treatment of the same duration (8P-8 min) but with low TCSI gives no PN (0% PN in Ducibella et al., 2002). Thus, some Ca^{2+}-sensitive events, like PN formation and CG exocytosis, can be stimulated by sustained and elevated $[Ca^{2+}]_{cyt}$. Moreover, our study demonstrates that these morphological events do not require that $[Ca^{2+}]_{cyt}$ be sustained and elevated during a period of 3 h after the onset of activation, whereas this is required when repetitive mode of signaling (Ducibella et al., 2002) or fertilization (Xu et al., 2003) is applied.

Although our results demonstrate that prolonged exposure to high $[Ca^{2+}]_{cyt}$ is still compatible with development, PN and implantation success are significantly better with oscillatory (24P-8 min) than sustained $[Ca^{2+}]_{cyt}$ signaling (20P-2 min). For example, this difference is even more prominent after multiplying the ratio of % PN (94%/81% for 8P-8 min and 20P-2 min) times the ratio of % implantation sites (72%/48% for 24P-8 min and 20P-2 min) (Tables 2 and 4), resulting in a 74% total increase in embryo success for fertilization-like oscillatory signaling. The question of whether events such as protein folding, chromatin remodeling, or zygotic gene activation are affected by events that occur shortly after fertilization and may be ultimately responsible for the decrease in implantation sites at later stages remains to be elucidated.

The concept of TCSI (Materials and methods) may help to explain species differences in the $[Ca^{2+}]_{cyt}$ signal at fertilization. Monotonic or oscillatory signaling at fertilization is utilized by many different organisms (Stricker, 1999). For example, the monotonic $[Ca^{2+}]_{cyt}$ increase in the fertilized amphibian and fish egg (also arrested at MII) has a longer duration (~15 min) and a higher amplitude (Stricker, 1999) than the individual $[Ca^{2+}]_{cyt}$ increases in the mammalian egg (Miyazaki et al., 1993). This combination of greater duration and amplitude may represent a different evolutionary strategy to generate a sufficient amount of CaMKII activity to complete the much shorter meiotic cell cycle time of these eggs than that of mammalian eggs. CaMKII activates the APC, degradation of cyclin B, and down-regulation of MPF activity necessary for MII exit (Lorca et al., 1993; Nixon et al., 2002; Tatone et al., 2002). In fertilized mammalian eggs, the extent of CaMKII activity is tightly linked to $[Ca^{2+}]_{cyt}$ (Markoulaki et al., 2003, 2004) and cyclin degradation is sensitive to the $[Ca^{2+}]_{cyt}$ (Nixon et al., 2002).

Autonomous CaMKII activity (Materials and methods) is the only process that is not always positively regulated by the duration of the $[Ca^{2+}]_{cyt}$ signal. Activity is down-regulated when the duration of $[Ca^{2+}]_{cyt}$ is extended to the 10th EF pulse (Fig. 13). Although the mechanism of this down-regulation is unknown, our results indicate that continuous Ca^{2+} stimulation causes a decrease in the amount of activation-competent enzyme. Because the Ca^{2+} sensitivity of CaMKII is not lost after 1 h post-fertilization (Markoulaki et al., 2004; Nixon et al., 2002; Tatone et al., 2002), oscillatory $[Ca^{2+}]_{cyt}$ signaling may provide an important mechanism to maintain normal levels of CaMKII sensitivity to Ca^{2+} for CaMKII-dependent late events of egg activation that are hitherto unidentified. In this study, the decrease in H1K activity required additional Ca^{2+} stimulation after the down-regulation of CaMKII activity; this suggests than non-autonomous CaMKII activity (not detected in the assay) or CaMKII-independent pathways are involved in MPF inactivation.

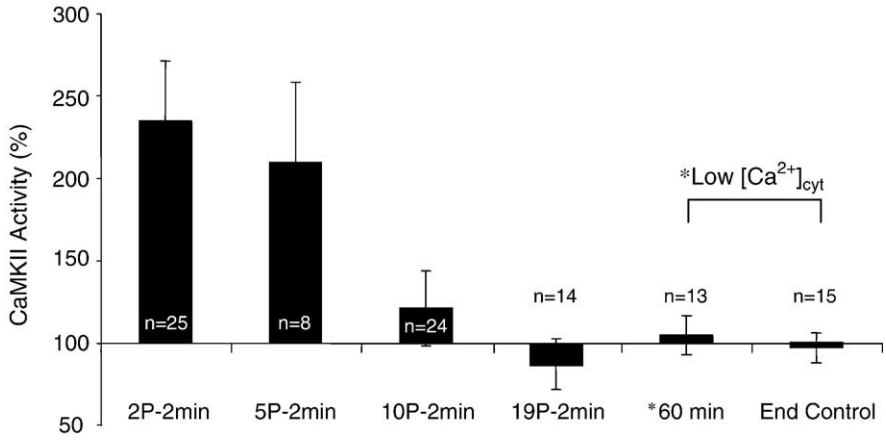

Fig. 13. CaMKII activity in relation to the duration of the Ca^{2+} stimulation. Mean CaMKII activity (±SEM) from eggs frozen within 1 min of the highest increase in $[Ca^{2+}]_{cyt}$ induced by the treatment and time indicated below each bar. *Low $[Ca^{2+}]_{cyt}$ refers to the Ca^{2+} level in untreated end controls or samples taken at 60 min in Fig. 5C (i.e., 20 min after the end of the 20P-2 min treatment). Activity is expressed relative to that in untreated eggs (100%). Control eggs were frozen at the beginning of each experiment and were assayed on the same day as the treated eggs in that experiment. Total number of determinations (*n*) are shown inside or above each bar (3 eggs per determination).

The amplitude of the $[Ca^{2+}]_{cyt}$ increase induced by the series of electrical pulses is estimated to be under the saturation level of Fura-2 (e.g., <1 μM, Fig. 6) and is not toxic (Nicotera and Orrenius, 1998) when its duration is prolonged beyond 50 min because activated eggs undergo normal cell division. Although chromosome movement stops temporarily when a high level of Ca^{2+} is injected (10–20 μM final concentration) into sea urchin eggs (Kiehart, 1981), mouse eggs subjected to a sustained $[Ca^{2+}]_{cyt}$ at ~1 μM during 50 min complete anaphase and form PN. Because this does not result in apoptosis or necrosis, the sustained level of high $[Ca^{2+}]_{cyt}$,which is thought to be a death trigger, does not appear to activate cell death pathways (Duchen, 2000).

A Ca^{2+} uptake process that is functionally linked to CICR in mouse egg

The dissimilar responses of unfertilized and fertilized eggs to a series of extracelluar Ca^{2+} influxes reveal important differences in their Ca^{2+} release and Ca^{2+} reuptake systems. Extracellular Ca^{2+} influx into fertilized eggs causes a relatively prolonged elevation of $[Ca^{2+}]_{cyt}$ immediately followed by a rapid and efficient Ca^{2+} uptake. In contrast, the same Ca^{2+} influx into unfertilized eggs causes neither a prolonged elevation of $[Ca^{2+}]_{cyt}$ immediately after the peak nor a rapid Ca^{2+} uptake. Because the CICR process (conferring Ca^{2+} sensitivity) is not activated in unfertilized eggs (Swann and Ozil, 1994), introduction of Ca^{2+} from outside is not capable by itself of activating an efficient Ca^{2+} uptake mechanism that is seen after fertilization or $InsP_3$ injection.

It is possible that in the absence of Ca^{2+} release from intracellular stores, any additional influx of extracellular Ca^{2+} cannot be recaptured by the ER due to the concentration of intraluminal Ca^{2+}. The Ca^{2+} in the ER is known to influence the kinetics of intracellular Ca^{2+} release and reuptake (Caroppo et al., 2003). But Ca^{2+} release from ER does not always imply that cytosolic ions are systematically and rapidly recaptured by the ER due to the decrease of intraluminal Ca^{2+}. For example, in sea urchin eggs, although fertilization causes Ca^{2+} release from the ER through a CICR mechanism (Lee and Shen, 1998; Swann et al., 1992; Whalley et al., 1992), the release of Ca^{2+} from the ER is neither followed by rapid uptake nor spontaneous oscillations (Lee and Shen, 1998; Whalley et al., 1992). In addition, in fertilized mouse eggs, despite the series of large Ca^{2+} influxes imposed every 2 min over the course of 40 min, Ca^{2+} reuptake is always completed in less than 2 min with a relative constant and short decay time (results herein). Thus, despite the large amount of excess Ca^{2+} provided by every electrical stimulation that results in overloading the egg ER (Ozil, 1998), the uptake mechanism is always efficient and capable of sequestering cytosolic Ca^{2+} with the same dynamics. Therefore, the extent to which the ER is filled to capacity may not be the direct trigger for recapturing Ca^{2+}. In fact, in mammalian eggs, there appears to be a none-or-all uptake mechanism that is not directly regulated by the extent of filling in the ER lumen.

Our results clearly suggest that robust intraluminal processes couple Ca^{2+} release and uptake. Redox mechanisms, such as those recently described by Li and Camacho (2004) and Higo et al. (2005) in *Xenopus* oocytes and somatic cells, fit well with this all-or-none process. These authors demonstrated that 2 luminal oxido-reductases, ERP 44 (Higo et al., 2005) and ERP 55 (Li and Camacho, 2004), which are both sensitive to the intraluminal redox state, differentially regulate the closing of the inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the activity of the sarcoplasmic ER calcium ATPases (SERCA) pumps, respectively. We describe here a similar functional dependency of Ca^{2+} uptake to Ca^{2+} release mechanisms. Therefore, if CICR is an important phenomenon at fertilization, we show that Ca^{2+} uptake plays an essential role in controlling the duration of the Ca^{2+} signal because it rapidly terminates the signal.

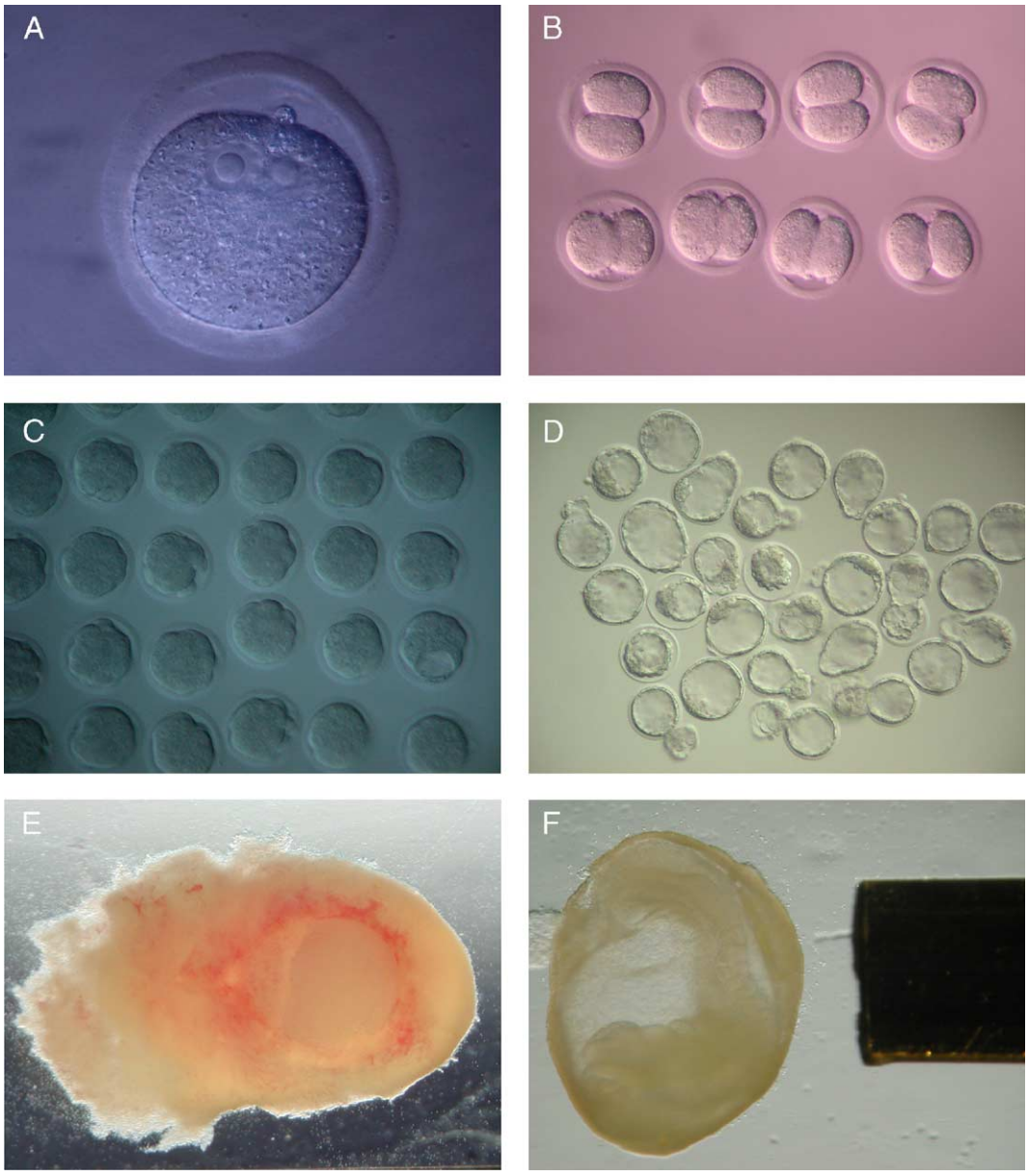

Fig. 14. Development of diploid parthenogenetic embryos subjected to 20P-2 min. (A) Typical activated one-cell embryo. Note the formation of two PN. (B) Two-cell stage parthenogenetic embryos. Note the regularity of the cleavage pattern. (C) Typical day 3 parthenogenetic morulae at the compaction-early-blastocyst stages. (D) Hatching blastocysts. (E) Implantation site at day 8 of pregnancy with the development of fetal membranes. (F) Early somite stage fetus inside of its envelope in the process of resorption.

Impact of Ca^{2+} parameters on development

The impact of Ca^{2+} signaling has been usually approached by focusing on the amplitude and the frequency modulation of the $[Ca^{2+}]_{cyt}$ signal. Activation with a shorter monotonic rise in $[Ca^{2+}]_{cyt}$ in aged eggs (Cuthbertson, 1983; Whittingham, 1980) is related to decreases in MPF and MAPK activities (Ducibella, 1998; Xu et al., 1997). The present study reveals that the time

Table 3
In vitro development up to D5[a] in response to Ca^{2+} treatments

Treatment	# Replicates	# Eggs	# D5 blastocyst
24P-8 min	6	192	146 (76%)
10P-2 min	3	62	49 (79%)
20P-2 min	3	85	64 (75%)

[a] D5 = Day 5.

Table 4
Post implantation development at D8[a] in response to Ca^{2+} treatments

Treatment	# Replicates	# 2 Cell eggs transplanted	# Implantation sites at D8	# Fetuses
24P-8 min (Ca^{2+})	6	50	36 (72%)[b]	0 (0%)
24P-8 min (Ca^{2+} + $InsP_3$)	13	141	99 (70%)[b]	0 (0%)
10P-2 min	4	40	18 (45%)[c]	2 (11%)
20P-2 min	7	67	32 (48%)[c]	1 (3%)

Groups with different letters were significantly different (χ^2 $P \leq 0.017$).
[a] D8 = Day 8.

component of the $[Ca^{2+}]_{cyt}$ signal has more biological importance than previously thought in fresh eggs. In the case of fertilization, it is possible that the initial $[Ca^{2+}]_{cyt}$ transient lasting 3 to 7 min has an impact on early events of fertilization, including chromatin remodeling and zygotic genome activation that is greater than previously thought. The approach described here makes it possible to uncouple dynamically the fluctuations of $[Ca^{2+}]_{cyt}$ and its ER counterpart in living cells. Our findings emphasize the functional importance of the time duration during which $[Ca^{2+}]_{cyt}$ is elevated, the resilience of the egg to prolonged EF-delivered Ca^{2+}, and the role of the rapid Ca^{2+} uptake process. In addition, they show that the mammalian oocyte can respond to a large range of $[Ca^{2+}]_{cyt}$ signal parameters. Future studies will address whether the dynamics of cytosolic and luminal $[Ca^{2+}]$ fluctuations (Banrezes et al., 2004) have long-term consequences that become manifest post-implantation.

In summary, the methodology described here makes it possible to drive the dynamic equilibrium of Ca^{2+} flux between the lumen and the cytosolic compartment in real-time in living eggs (Banrezes et al., 2004) and will provide a powerful system to examine long-term effects of Ca^{2+} signaling that occur during fertilization.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.02.035.

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