Egg activation is the result of calcium signal summation in the mouse

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Abstract

Egg activation in mammals is caused by cytosolic Ca2+ oscillations that are essential for development. However, despite increasing knowledge about signal transduction mechanisms, the functional linkage between frequency, amplitude and duration of the Ca2+ signal and the kinetics of pronucleus formation has not yet been defined. While a wide range of Ca2+ signal parameters are efficient in causing egg activation, the basic rules governing how the egg integrates these signalling events are not yet clear. Thus, in the perspective of better understanding how the egg processes Ca2+ signalling events, the objective of this study was to determine experimentally whether the efficiency of egg activation and the subsequent early developmental stages rely on Ca2+ summation signalling. Non-fertilized, but freshly ovulated mouse eggs, were subjected to a series of repetitive Ca2+ influxes of various patterns modulated by a non-invasive membrane electropermeabilization method. Using a combination of two suboptimal treatments we have shown that mouse eggs can sum up the effects caused by various patterns of intracellular Ca2+ concentrations transient during the period of egg activation. In addition, overloading the intracellular milieu by repetitive Ca2+ influxes did not seem to inhibit the process of activation. The kinetics of pronuclear formation among a population of eggs treated in the same conditions became accelerated when the total dose of Ca2+ signal ‘experienced’ by the eggs was increased. The results suggested that summation of the biological effects of all Ca2+ signals constitutes an important mode of Ca2+ signal integration.

Introduction

In mammals, egg activation at fertilization is induced by a series of repetitive increases in intracellular Ca2+ concentration ([Ca2+]i), due to Ca2+ release from the endoplasmic reticulum (MiyaZaki et al. 1993, Kline & Kline 1994, Swann & Parrington 1999). These Ca2+ oscillations are essential for development (Ozil 1990, Kline & Kline 1992, Bos-Mikich et al. 1997, Ozil & Huneau 2001). They begin a few minutes after gamete fusion (Cuthbertson 1983, Lawrence et al. 1997, Deguchi et al. 2000), occur at various frequencies and cease at the time of pronucleus (PN) formation, i.e. 4–6 h later (Jellerette et al. 2000, Marangos et al. 2003). However, despite increasing knowledge about signal transduction mechanisms, the functional significance of these signalling events and the rules according to which Ca2+ signals are processed, such as frequency encoding or spike counting (Berridge 1997), or simply the summation of the biochemical effects caused by a train of [Ca2+]i transients, are not very clear. It has been shown that mammalian oocytes have the potential to decode a wide range of Ca2+ signal regimens. For example, if the number of Ca2+ oscillations can regulate the early events of egg activation (Vitullo & Ozil 1992, Lawrence et al. 1998, Ducibella et al. 2002) as well as pre- and post-implantation development of parthenotes (Ozil & Huneau 2001), a single and large monotonic increase in the [Ca2+], (intracellular Ca2+ concentration) can also activate the egg and promote parthenogenetic preimplantation development (Cuthbertson 1983, Surani et al. 1984, Ozil et al. 2005). It has recently been shown that after 25 or 50 min of elevated cytosolic Ca2+, activated eggs readily develop to the blastocyst stage with no sign of apoptosis or necrosis. Some of them implant. The importance of the duration of Ca2+ elevation, whether continuous (Ozil et al. 2005) or oscillatory (Ducibella et al. 2002), suggests that the egg sums up the Ca2+ signal input over time. Thus, although eggs have a surprising degree of tolerance for a prolonged change in the [Ca2+], it is important to know if ‘summation’ of biochemical effects is a functional rule governing how the egg integrates Ca2+ signalling events over the period of egg activation.

In order to better reveal such possible functioning, we have set up a global approach with a non-invasive electropermeabilization technique which makes it possible to...
bypass the self-regulated process of Ca$^{2+}$ signalling triggered by fertilization. This artificial means gives us the possibility of dosing the Ca$^{2+}$ ion influx at will by modulating either the voltage amplitude of the electrical pulse or the number of pulses without causing Ca$^{2+}$ release from the endoplasmic reticulum (ER) (Ozil et al. 2005). In practice, we have designed two treatments with a poor capability to activate the freshly ovulated eggs but with different and complementary patterns. The first treatment consisted of a single but large Ca$^{2+}$ signal which lasts about 10 min while the second consisted of a series of six small Ca$^{2+}$ signals given at 30-min intervals. Since these two treatments have poor efficiency when they are considered individually, it becomes possible to reveal the capacity of the eggs to sum up the effects of the two treatments when they are applied successively to a common group of eggs.

Results clearly suggested that freshly ovulated mouse eggs are capable of summing up the biochemical effects caused by the Ca$^{2+}$ influx during the period of egg activation and it appears that eggs become activated when the total Ca$^{2+}$ signal input (TCSI) ‘experienced’ by the egg reaches a minimal threshold.

This new insight into the egg’s capability to transduce Ca$^{2+}$ signalling associated with the development of new experimental techniques for driving the kinetics of Ca$^{2+}$ signalling on a population of eggs will provide new possibilities for using Ca$^{2+}$ signalling to better understand the dynamics of oocyte functioning and its impact on the developmental processes.

Materials and Methods

Eggs

F1 (C57BL/6 × CBA) hybrid female mice were superovulated at 8–9 weeks of age by an intraperitoneal injection of 8 IU pregnant mare serum gonadotrophin (Folligon; Intervet, Angers, France) followed 48–50 h later by a treatment with 7.5 IU human chorionic gonadotrophin (hCG; Choloron; Intervet). At 13–13.5 h post-hCG, unfertilized eggs were flushed from the oviducts and collected in Hepes-buffered M2 medium (Sigma). Cumulus cells were removed by brief exposure to 0.1% hyaluronidase (Sigma) and cultured in M16 (Sigma). The unfertilized eggs were then subjected to repetitive calcium stimulation 13.5–14.5 h after hCG administration (median value: 14 h) in the presence of 0.3 μg/ml cytochalasin D (Sigma) to prevent the extrusion of the polar body with the second set of haploid monovalent chromosomes. For control experiments, fertilized eggs were obtained from superovulated F1 females mated to F1 males.

Activation procedure and intracellular Ca$^{2+}$ measurement

Freshly ovulated eggs were placed in a micro-chamber that allows for both electric field (EF) pulses and intracellular Ca$^{2+}$ imaging (Ozil & Swann 1995). Briefly, a 10-s wash in isotonic and low ionic strength medium, 0.3 M glucose (Aristar; BDH, Poole, Dorset, UK) and 0.1 mM Ca$^{2+}$ allows a rapid diffusion of the ionic content across the periviteline space prior to electrical pulsation. Next, following an EF pulse which causes a transmembrane Ca$^{2+}$ ion influx, a rapid wash with M16 culture medium ensured membrane healing and blockage of the Ca$^{2+}$ influx. The rapid succession (a few hundred ms) of these two washing steps makes it possible to adequately control calcium influx on an entire batch of eggs simultaneously. The EF pulse was made up of a series of alternative pulses of 45 μs at 5-μs intervals for 300 μs. Under such conditions, eggs were subjected to various numbers of EF pulses during the treatment. Three voltage amplitudes, 1.62 kV/cm, 1.42 kV/cm and 1.12 kV/cm, were used in order to graduate the transmembrane Ca$^{2+}$ influx and make it possible to conjugate large and small Ca$^{2+}$ influxes during the same treatment. For the measurement of [Ca$^{2+}$]c, eggs were incubated for 15 min at 37°C with 20 μM Fura 2-AM (Molecular Probes, Leiden, The Netherlands). The dye was first dissolved in DMSO containing the detergent Pluronic F-127 (Molecular Probes), final concentration 0.08% (v/v) and 0.016% (w/v) respectively, and then diluted in M16 medium. The eggs were placed on the stage of a Nikon TE2000 (Nikon France, Champigny sur Marne, France) inverted microscope fitted with a Fluor 100/1.3 oil objective. The individual eggs were held in the micro-chamber by micropipette as previously described (Ozil & Swann 1995). The optical field was illuminated with a 75 W xenon arc lamp and wavelengths were selected at 340 ± 5 nm and 380 ± 5 nm using a Cairn Optoscan monochromator (DIPSI 92 325, Chatillon, France). A long pass emission filter (upper 520 nm; Nikon) was used to transmit emitted light from the Fura 2 fluorochrome to a Photonic science extended ISIS video camera (DIPSI 92 325, Chatillon, France). The emission was recorded at 340 and 380 nm wavelengths every second and intracellular Ca$^{2+}$ levels were displayed in terms of a ratio of fluorescence (F340/F380) which increases with rising [Ca$^{2+}$]c. The whole process was controlled by Axon Imaging Workbench software v2.2.1 (Axon, Union City, CA, USA).

Treatment of eggs

The strategy of this study relies on the use of four treatments that are described here. The first treatment was made up of four EF pulses of high amplitude (HA means 1.62 kV/cm) delivered at 2-min intervals to cause a prolonged intracellular Ca$^{2+}$ increase lasting about 10 min, as shown in Fig. 1. This treatment is named ‘4HA-2 min’. After the treatment, the eggs were removed from the micro-chamber and placed under culture conditions. The second treatment, named 6LA-30 min, was made up of six EF pulses of low amplitude (LA means 1.12 kV/cm) given at 30-min intervals. The total duration of the treatment did not exceed the
time-period that elapsed between the metaphase II stage and the PN formation. The profile of \([Ca^{2+}]_\text{cyt}\) change is shown in Fig. 2. The third treatment, named 4HA + 5LA, consisted of the fusion of the two previous treatments, the 4HA-2 min and five pulses taken from the 6LA-30 min. This treatment caused an initial \(Ca^{2+}\) increase of large amplitude and duration followed by a series of five pulses of lower amplitude and shorter duration given at 30-min intervals, as shown in Fig. 3. The fourth treatment was designed to maximize the \(Ca^{2+}\) influx during the initial 3-h period of activation. This treatment was made up of a large \([Ca^{2+}]_\text{cyt}\) change resulting from five EF pulses of 1.62 kV/cm amplitude given at 2-min intervals followed by 19 EF pulses of 1.42 kV/cm amplitude given at 8-min intervals. This treatment was named 5HA +19P-8 min (Fig. 4).

Control experiment

The potential bias of our methodology was tested by subjecting fertilized eggs collected at the time of PN formation to an electric pulse of 1.42 kV/cm every 8 min for 3 h in glucose solution but in the absence of \(Ca^{2+}\) ions. Treated fertilized eggs were transferred to the two-cell stage to pseudopregnant recipients to record the rate of survival to term.

Graphical estimate of the TCSI

In order to compare the degree of \(Ca^{2+}\) stimulation between treatments, the TCSI of a given treatment was estimated by summing up the ratio data points for all records according to the method previously described (Ozil et al. 2005). In brief, the TCSI was generated by summing the fluorescence ratio (340/380) over time for the four records shown in Figs 1–4. 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 SigmaPlot 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 'experienced' by the egg during the time-course of the treatment. Since \(Ca^{2+}\) influx in unfertilized eggs is not capable of causing \(Ca^{2+}\) release from intracellular stores (Ozil et al. 2005) and the rate of acquisition is one point per second, the TCSI is equivalent to the total area of the cytosolic change induced by the sole \(Ca^{2+}\) influx from outside.

Culture and observation of treated eggs

Eggs were kept in M16 medium complemented with 0.3 µg/ml cytochalasin-D under 5% CO₂ at 37°C and observed every hour to determine the time and the dynamics of pronuclear formation. Eggs with PN were scored using an inverted microscope equipped with Nomarski optics (Nikon). After the final observation, eggs were washed three times in cytochalasin-free M16 and then some of them were cultured in vitro for 5 days. The number of blastocysts formed was counted at day 5 (Table 1).

Embryo transfer and autopsy

Since development in vivo is generally regarded as being preferable in most regards to development in vitro, we decided to undertake an evaluation of the developmental potential of diploid parthenogenetic embryos cultured in vitro for 1 day and then transplanted into pseudopregnant recipients. Each recipient received ten diploid parthenogenetic embryos at the two-cell stage in the left oviduct. The recipients were autopsied at day 8 of pregnancy (day 0 being the day of the transfer) and the implantation sites were counted.

Statistical evaluation

The data were plotted using the SigmaPlot 2001 software package (SPSS Schimmebuschstr 25 D-40699, Erkath).
Statistical significance was assessed using the $\chi^2$ test; $P < 0.05$ was considered to be statistically significant.

Results

Profile of the $[\text{Ca}^{2+}]_{\text{cyt}}$ changes caused by the four experimental treatments

The 4HA-2 min treatment was designed to assess the impact of a large $\text{Ca}^{2+}$ increase on freshly ovulated eggs. The four high amplitude electrical pulses (1.62 kV/cm) given at 2-min intervals bring the four $\text{Ca}^{2+}$ influxes closer, thus preventing rapid restoration of the resting level, as previously shown (Ozil et al. 2005). Instead, a prolonged increase in the cytosolic $\text{Ca}^{2+}$ concentration lasting about 10 min was generated, as can be seen in Fig. 1. In contrast, the 6LA-30 min treatment was designed to assess the impact of a series of six smaller $\text{Ca}^{2+}$ signals given every 30 min for 3 h. The voltage amplitude was reduced to 1.12 kV/cm and the duration of every $[\text{Ca}^{2+}]_{\text{cyt}}$ change was about 2 min. We can see in Fig. 2 that whatever the rank of the electrical pulses in the series, the pattern of the $[\text{Ca}^{2+}]_{\text{cyt}}$ changes appeared to be similar. Moreover, it can be seen that no additional signal due to spontaneous $\text{Ca}^{2+}$ release from intracellular stores occurred between electrical pulses. When these two treatments were simply run successively at 30-min intervals, we can see from the record in Fig. 3 (4HA + 5LA) that the whole profile resembled the summation of the profiles in Figs 1 and 2. The 5HA + 19P-8 min treatment was designed to provide an excess of $\text{Ca}^{2+}$ influx throughout the period of egg activation, i.e. the first 3 h of the developmental process. This treatment caused an initial large $[\text{Ca}^{2+}]_{\text{cyt}}$ change which lasted more than 15 min, followed by a series of 19 shorter duration $[\text{Ca}^{2+}]_{\text{cyt}}$ changes.

Table 1  Egg activation and developmental potential in relation to the activation treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of activated eggs</th>
<th>Rate of blastocysts at day 5</th>
<th>Rate of implantations sites at day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HA-2 min</td>
<td>6%a (4/67)</td>
<td>75% (3/4)</td>
<td></td>
</tr>
<tr>
<td>6LA-30 min</td>
<td>48%b (114/240)</td>
<td>74% (28/38)</td>
<td>66% (33/50)</td>
</tr>
<tr>
<td>4HA+5LA</td>
<td>89%c (220/247)</td>
<td>62% (75/121)</td>
<td>90% (45/50)</td>
</tr>
<tr>
<td>5HA + 19P-8 min</td>
<td>99.6% (227/278)</td>
<td>71% (32/45)</td>
<td>88% (53/60)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>90% (54/60)</td>
<td>80% (16/20)</td>
</tr>
</tbody>
</table>

Values with a different superscript differ significantly ($P < 0.05$).
The rate of activation of eggs of 4HA-2 min, 6LA-30 min and 4HA+5LA treatments were significantly different from each other.

The rate of blastocyst formation at day 5 was not significantly different between the treatment, but the rate of parthenogenetic blastocysts was significantly lower in the control. The implantation rate of the parthenogenetic embryos subjected to 6LA-30 min was significantly lower than the 4HA+5LA and 5HA-19P-8 min treatments.
The TCSI

The graphical representation of the TCSI (see Materials and Methods) delivered by the treatment integrates the amplitude of the $[\text{Ca}^{2+}]_{\text{cyt}}$ and the duration of the change (Fig. 5). Because the records taken for these plots had similar ratio amplitudes (Figs 1–4) their respective TCSI gave a reasonably good representation of the total Ca$^{2+}$ signal during which the eggs were exposed to high $[\text{Ca}^{2+}]_{\text{cyt}}$. Hence, the TCSI from the 4HA-2 min treatment appears similar to the one delivered by the six pulses from the 6LA-30 min treatment. The TCSI of the 4HA + 5LA treatment was equivalent to the sum of the TCSI from the 4HA-2 min and 6LA-30 min treatments but three times lower than the TCSI of the 5HA + 19P-8 min treatment. Such graphical representation of the TCSI made it possible to explore the relationship between the total Ca$^{2+}$ changes during the time-course of the treatment and the egg response.

Rate and time-course of egg activation

For all treatments, all eggs survived but the rate of egg activation and the pronuclear time-course varied. The least efficient treatment was the 4HA-2 min as only four out of 67 treated eggs were capable of forming two PN (rate of activation 6%). In this experiment, the formation of the PN began 6 h after the treatment for the earliest egg and finished 8 h later (Fig. 6). The rate of egg activation caused by the 6LA-30 min treatment was higher (48%) but still remained suboptimal. The time-course of PN formation was also slightly accelerated, as can be seen in Fig. 6. It began 5 h after the first electrical pulse for the earliest egg and finished 9 h later. However, when eggs were subjected successively to the two previous treatments, i.e. 4HA + 5LA, the rate of egg activation reached 89% and the formation of the PN was accelerated.

It began 4 h after the first electrical pulse and finished 9 h later (Fig. 6). In contrast, when the Ca$^{2+}$ influxes were maximized by the 5HA + 19P-8 min treatment almost all eggs were activated (99.6%) and formed PN 3 h after the first pulse for the earliest and 5 h for the latest.

Developmental potential

In this study, almost all parthenogenetic eggs were capable of developing in vitro and reaching the blastocyst stage at a high rate (see Table 1). We could not detect any significant developmental differences in relation to the treatment. However, when parthenogenetic eggs were transferred at the two-cell stage into the oviduct of pseudopregnant recipients, we found that the implantation rate was always maximized when eggs were subjected to a high TCSI. The eggs subjected to the 4HA-5LA treatment had an implantation rate significantly higher than eggs subjected to the 6LA-30 min treatment (90% versus 66% respectively). When eggs were subjected to a higher TCSI treatment, such as the 5HA + 19P-8 min, the implantation rate remained high (88%).

Control experiment

The control experiment revealed that fertilized eggs subjected to sequential washing in glucose and an electric pulse of 1.42 kV/cm every 8 min for 3 h in the absence of Ca$^{2+}$ influx were capable of developing in vitro to the blastocyst stage at a high rate (90%, 54/60; Table 1). They also implanted at a high rate (80%, 16/20; Table 1) and were capable of giving birth to viable offspring at a high rate (75%, 45/60). It thus appears that neither repetitive electrical stimulation nor the series of sequential washings had any deleterious effect by themselves in the absence of Ca$^{2+}$ influx (Ozil & Swann 1995).

Discussion

In the course of the present study we uncovered two functional properties of mouse eggs during the process of activation. The first one concerned the capacity of eggs to sum up the physiological effects caused by $[\text{Ca}^{2+}]_{\text{cyt}}$ changes during the period of egg activation. The second one concerned the absence of inhibition of egg activation when eggs were subjected repetitively to high doses of Ca$^{2+}$ influx distributed at 8-min intervals over the first 3 h of the developmental process. Using a specific combination of two different but suboptimal activation treatments, we have here shown that freshly ovulated mouse eggs do not require a specific number, frequency or amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ signals to exit meiosis and enter the cell cycle if the total dose of Ca$^{2+}$ stands above a minimum threshold. These results open a series of new questions regarding the biological role of the signal dynamics of Ca$^{2+}$ and offer a few guidelines to designing experimental
approaches aimed at understanding the long-term effects of Ca\(^{2+}\) signalling during the process of egg activation.

The control of Ca\(^{2+}\) influx was ensured by a previously described micro assembly that combines micro fluidic techniques and membrane electroporation (Ozil & Swann 1995). In brief, the dosage of Ca\(^{2+}\) influx through the egg membrane was ensured by using a sequential micro washing of eggs before the electrical pulse with a non-conductive medium and very fast micro washing after the pulse with culture medium to preserve cell viability. The series of sequential washings and electrical shocks appeared to be non-invasive as the control cell viability. The series of sequential washings and electrical shocks with culture medium to preserve cell viability. The series of sequential washings and electrical shocks appeared to be non-invasive as the control cell viability. The series of sequential washings and electrical shocks appeared to be non-invasive as the control cell viability. The series of sequential washings and electrical shocks appeared to be non-invasive as the control cell viability.

A high rate of activation was obtained when mouse eggs were subjected successively to two treatments, each of which provided a poor rate of egg activation. We have exploited the possibilities offered by our technique to design two suboptimal treatments. The 4HA-2 min treatment provided a single but prolonged \([\text{Ca}^{2+}]_{\text{cyt}}\) increase that is not capable of giving a high rate of egg activation (6%, see Table 1) while the 6LA-30 min treatment exploited the possibility of subjecting unfertilized eggs to a series of six smaller Ca\(^{2+}\) influxes imposed at 30-min intervals (Fig. 2). In this case, the rate of egg activation reached 48% (see Table 1) but remained suboptimal. If we consider the graphical estimate of the total Ca\(^{2+}\) signal input of these two treatments (TCSI; Fig. 5), we can see that the TCSI of the 4HA-2 min treatment appeared to be similar to the TCSI of the 6LA-30 min treatment, the results of which confirm previous findings that a single Ca\(^{2+}\) signal is generally inefficient at causing egg activation on freshly ovulated eggs (Cuthbertson 1983, Ducibella 1998).

In contrast, when eggs were subjected successively to the 4HA-2 min treatment and to five pulses from the 6LA-30 min treatment (i.e. 5LA-30 min), the steep increase in the rate of egg activation to 89% revealed some interesting features on how the egg decodes the pattern of Ca\(^{2+}\) signalling. This increase in efficiency cannot be attributed to any frequency change because the period of time between pulses was kept equal to 30 min. Nor can it be specific to some particular \([\text{Ca}^{2+}]_{\text{cyt}}\) amplitude as the maximal amplitudes caused by the two treatments (4HA-2 min and 4HA-5LA) were similar (Figs 1 and 3). We can, however, see that the activation rate of eggs subjected to the 4HA-2 min and to the 5LA-30 min treatments (89%) went far above the simple addition of the rates of egg activation provided by each individual treatment (i.e. 6% and 48%; \(P < 0.05\)). Therefore, the biological impact of the series of six small pulses (6LA-30 min) is enhanced by the initial large Ca\(^{2+}\) increase (4HA-2 min) given before it. It is quite possible that this series of small Ca\(^{2+}\) signals given after the initial one had prevented a rapid rebound of MPF (maturation promoting factor) activity that would have stopped the decondensation of the chromatin (Collas et al. 1993, Ducibella et al. 2002). Nevertheless, this steep increase in activation efficiency revealed that egg activation occurs when the TCSI goes beyond a minimal threshold. The TCSI of the large initial Ca\(^{2+}\) increase in the 4HA-2 min treatment was not high enough to cause a high rate of egg activation. It has been shown that the large initial Ca\(^{2+}\) increase has to be prolonged beyond 50 min to cause a high rate of egg activation (Ozil et al. 2005). But when a series of small signals was added (6LA-30 min), the rate of egg activation jumped from 6% to 89%. Therefore, the downstream physiological Ca\(^{2+}\) effects appear to be additive and Fig. 5 clearly shows that every increase of the TCSI is followed by an increase in the rate of egg activation and a shorter PN time-course.

Some evidence that summation of Ca\(^{2+}\) signals regulates egg activation can also be drawn from several other studies. Ducibella et al. (2002) have shown that, in the course of a 24-pulse treatment activating nearly all mouse eggs, the completion of many events of activation is dependant on the number of Ca\(^{2+}\) transients. Direct visualization of cyclin B1 turnover showed that the first calcium transients in fertilized mouse eggs induced discrete reductions in the cyclin B1 level that are additive (Nixon et al. 2002). However, if the frequency is too low, activation may not be optimal as cyclin levels may rebound due to continued cyclin synthesis (Nixon et al. 2002) and egg activation would be compromised. So, whatever the pattern of the Ca\(^{2+}\) regime, mouse eggs appear to be capable of piling up the biochemical changes provided by any \([\text{Ca}^{2+}]_{\text{cyt}}\) change and committing themselves toward

Figure 6 Kinetics of pronuclear formation for eggs subjected to parthenogenetic treatments. For each individual experiment the percentages of eggs at the PN stage were scored at each hour after the first electrical pulse. The graph points represent the mean of at least three replicates ± S.E.M. Replicates of a given treatment were carried out on similar numbers of eggs.
the pronuclear stage if the total dose of Ca$^{2+}$ to which they are subjected is sufficient (Ozil et al. 2005).

**Is this summation effect inhibited by intracellular Ca$^{2+}$ overloading?**

In order to identify the upper limit beyond which this summation process becomes inefficient, we tested the capacity of eggs to integrate higher doses of Ca$^{2+}$ signals by subjecting them to increasing numbers of Ca$^{2+}$ stimulations over the same time frame, i.e. 3 h. The eggs subjected to the 5HA + 19P-8 min treatment showed a high rate of activation (99.6%). If we consider the dynamics of pronuclear formation as phenomenological criteria that sum up the global biochemical activity of the egg, we can see that increasing the dose of the Ca$^{2+}$ load always results in faster kinetics of PN formation with no apparent inhibiting effect due to the increase in frequency, the number of stimulations, the duration of the treatment or the increased dose of the Ca$^{2+}$ influx. Still, we can see that among eggs subjected to the same treatment, some responded faster than others (Fig. 6). This variability in kinetics might originate in the capability of various eggs to process the Ca$^{2+}$ influx as well as in differences in the intensity of Ca$^{2+}$ influx experienced by each egg inside the chamber. Nevertheless, we see that increasing the number and the amplitude of pulses makes it possible to force an increased proportion of eggs to rapidly form PN in a shorter time-course with no sign of inhibition. A recent study has shown that completion of meiosis and fertilization-associated events, such as CG exocytosis, the dynamic of kinase activity and recruitment of mRNAs, can be graded in relation to the duration of a single Ca$^{2+}$ signal and that activated eggs readily develop to the blastocyst stage with no sign of apoptosis or necrosis (Ozil et al. 2005). Therefore, the present study has clearly show that some additional Ca$^{2+}$ signals can be imposed at any moment during the period of egg activation to maximize the rate of egg activation. The upper limit of the Ca$^{2+}$ dose that inhibits the process of egg activation remains to be determined.

**Conclusions**

With regard to the egg responses that we obtained in the present study, some general conclusions can be drawn on the way eggs integrate Ca$^{2+}$ signalling.

i) The reduction of kinetic variability when eggs are subjected to higher doses of Ca$^{2+}$ stimulation means that faster eggs, those which form the PN early, are not inhibited by subsequent pulses while the slower eggs remain capable of integrating Ca$^{2+}$ ions and accelerating their dynamics. The absence of any inhibiting effect by subsequent Ca$^{2+}$ signals explains why 100% of eggs can be activated with our methodology.

ii) All freshly ovulated eggs are capable of forming a PN in a very limited time, i.e. from 3 to 4.5 h, if they receive enough Ca$^{2+}$.

iii) The Ca$^{2+}$ signal is the master regulator in these kinetics but since various patterns of Ca$^{2+}$ signal can activate freshly ovulated eggs, it is difficult to assume that a specific signature in terms of amplitude, frequency or number of Ca$^{2+}$ signal is critical for egg activation and early cleavage.

Despite this great flexibility in processing the Ca$^{2+}$ signal, the question remains as to whether the pattern of the Ca$^{2+}$ signal affects some epigenetic modifications during the activation period that impact the developmental processes at later stages. The results in Table 1 show that almost all parthenogenetic diploid eggs were capable of cleaving and forming typical blastocysts in vitro. The eggs subjected to the 4HA + 5LA and 5HA + 19P-8 min treatments and transplanted at the two-cell stage into foster mothers gave high rates of implantation at day 8 of pregnancy. It thus appears that if a freshly ovulated egg receives a sufficient amount of calcium influx from outside to be activated, it has the potential to go through all the preimplantation stages. Not even the higher doses of Ca$^{2+}$ delivered by the 5HA + 19P-8 min treatment inhibit implantation.

**Summary**

The present investigation has provided evidence that activation and preimplantation development of mouse eggs do not rely on a specific pattern of Ca$^{2+}$ signalling if the TCSI is sufficient. However, with regard to the potential post-implantation effects, optimal amplitudes, number and frequencies have yet to be fully established. Nevertheless, the present results support the hypothesis that egg activation occurs when the summation of multiple Ca$^{2+}$ stimuli reaches a sufficient threshold. The most prominent effect of the Ca$^{2+}$ regime is on the kinetics of egg activation which can be accelerated by increasing the cumulative exposure time to Ca$^{2+}$ influx during the 3 h of parthenogenetic stimulation. Hence, the flexibility and the fidelity with which mammalian eggs can respond to Ca$^{2+}$ signalling during egg activation open new possibilities for driving and exploring the remodelling mechanisms at work during the process of egg activation. Regarding the long-term consequences, complementary strategies are under development (Banerze et al. 2004) to further evaluate how the modulation of Ca$^{2+}$ signalling dynamics during fertilization might impact the remodelling of parental chromosomes.

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