Development 128, 917-928 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV3358

Activation of rabbit oocytes: the impact of the Ca^{2+} signal regime on development

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Accepted 6 December 2000; published on WWW 26 February 2001

SUMMARY

Postfertilization manipulation of mammalian embryos results in various developmental alterations. To determine whether the manipulation of the Ca^{2+} regime causing oocyte activation is a valuable experimental means in helping understand the biological process by which embryos integrate signals from outside and later regulate gene expression, we linked Ca^{2+} signal parameters i.e. amplitude, number and frequency, with the efficiency and quality of postimplantation development. Freshly ovulated rabbit oocytes were subjected to repetitive and modulated Ca^{2+} influx. The results provide three major pieces of information. Firstly, the Ca^{2+} stimulus is the most efficient signal activating mammalian eggs when it is applied in a repetitive manner, the amplitude being the crucial factor. Secondly, the dynamics of early cleavage does not appear to be determined by either the frequency or the amplitude of modulation of the Ca^{2+} signal that activates the oocyte. Thirdly, amplitude and temporal modulation of the Ca^{2+} signal in the early minutes influences the developmental performance and the morphology of the rabbit parthenogenetic conceptus at day 11.5 of pregnancy. The results demonstrate the importance of epigenetic events during postfertilization as well as the possible uses of Ca^{2+} modulation in studying long term developmental effects.

Key words: Ca^{2+} signal, Oocyte activation, Fertilization, Parthenogenesis, Rabbit

INTRODUCTION

In mammals considerable progress in understanding the fertilization processes (for review see Yanagimachi, 1994) has progressively opened up new horizons in the manipulation of mammalian eggs at earlier postfertilization stages (Seidel, Jr., 2000). However, recent results from a number of laboratories indicate that in vitro manipulation of gametes and embryos during the very earliest developmental stages can cause a variety in the long-term alterations of the developmental processes. It has been shown that in vitro culture causes alteration in the expression of the zygotic genome (Stojanov et al., 1999; Blondin et al., 2000; Niemann and Wrenzycki, 2000), and a great reduction in viability (Biggers and Papaioannou, 1991; Holm et al., 1996; Sinclair et al., 1999). Altered fetal growth and neonatal malformation after running nuclear transfer protocols are also well documented (Willadsen et al., 1991; Reik et al., 1993; Kruip and den Daas, 1997; Renard et al., 1999). At present, the underlying mechanisms responsible for these anomalies remain unknown but experimental manipulations during the very early stages reveal that the fertilization period displays critical sensitivity to external perturbation. This sensitivity to initial conditions (Swann and Ozil, 1994) contrasts with the highly efficient regulation capacity and survival that embryos show when they are split in vitro during the early stages of cleavage and transferred into recipients (Tarkowski, 1959; Moore et al., 1968; Willadsen, 1980; Willadsen, 1981; Ozil, 1983). A possible key to elucidating this apparent paradox – that is to say the embryo's robust survival rate to term in spite of in vitro manipulation and large scale reductions in cell numbers as of the first mitosis compared to its low level of resistance to external stimuli during the fertilization period – is to better understand the complex dynamics of molecular and cellular changes triggered by the incoming sperm.

In mammalian eggs, the first prominent physiological changes observed after fertilization take the form of repetitive increases in intracellular Ca^{2+}. These increases continue at various frequencies for 3-4 hours and stop around the time of pronucleus formation (Jones et al., 1995; Day et al., 2000) These Ca^{2+} rises are required to induce egg activation, which consists of a sequence of morphological and molecular events that include cortical granule exocytosis (Abbott et al., 1999), resumption of meiosis, (Collas et al., 1995) extrusions of the second polar body, formation of pronuclei, DNA synthesis and mitotic cleavage (Schultz and Kopf, 1995; Xu et al., 1997). Considerable knowledge on how sperm triggers Ca^{2+} oscillations has recently been gained. It has been shown that the fertilization process bears some similarity to the Ca^{2+} release mechanisms described for most somatic cells (Berridge and Irvine, 1984). The incoming sperm would provoke the activation of some form of phospholipase C which cleaves phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2), generating the second Ca^{2+}-releasing messenger, inositol 1,4,5-

trisphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3 leads to an increase in intracellular Ca^{2+} by stimulating the opening of its receptor, the Ins(1,4,5)P_3 receptor, which is located in the endoplasmic reticulum (Fissore and Robl, 1993; Dupont et al., 1996; Xu et al., 1997; Jones et al., 1998; Swann and Parrington, 1999) but the signalling pathways responsible for the increase in the Ca^{2+} Induced Ca^{2+} Release mechanism (CICR) are not yet fully understood. Nevertheless, the possibility of by-passing the natural mechanisms controlling fertilization-induced Ca^{2+} release from internal stores by direct Ca^{2+} transmembrane influx (Ozil and Swann, 1995) is of particular interest in studying how a precise range of amplitude or frequency signalling alterations can affect the developmental processes at later stages.

In somatic cells it has been shown that different patterns of Ca^{2+} increase can regulate the migration of transcription factors from the cytoplasm into the nucleus as well as the degree of gene expression (Fields et al., 1993; Berridge, 1997; Dolmetsch et al., 1998; Li et al., 1998). In mammalian eggs different patterns of Ca^{2+} increase during oocyte activation have been shown to drive activation and have an effect on the preimplantation development of the embryos (Cuthbertson and Cobbold, 1985; Ozil, 1990; Vitullo and Ozil, 1992; Ozil and Swann, 1995; Collas et al., 1995; Bos-Mikich et al., 1997) but the postimplantation effect of the amplitude and frequency of intracellular Ca^{2+} during activation as opposed to a monotonic rise is not clear since monotonic rises in Ca^{2+} have been shown to trigger parthenogenetic development up to day 30 in cattle (Fukui et al., 1992), day 24 in sheep (Loi et al., 1998) and day 29 in pig (Kure-bayashi et al., 2000).

Therefore, to determine whether the regime of Ca^{2+} signalling could offer a valuable experimental means for understanding the biological process by which embryos integrate signals during the postfertilization period and regulate both genome expression and the developmental pathway at later stages, the present study was undertaken to link Ca^{2+} signal parameters i.e. amplitude, number and frequency, with the postimplantation development of parthenogenetic diploid rabbit embryos.

We subjected freshly ovulated rabbit oocytes to repetitive Ca^{2+} influx, the amplitude of which was modulated by electroporation. We found that the amplitude, the number and the time interval of Ca^{2+} influx during oocyte activation affect postimplantation development.

MATERIALS AND METHODS

Media used

Oocytes were collected in M2 medium supplemented with 4 mg/ml of BSA (fraction V; Sigma #A-4503) and 320 units/ml of hyaluronidase (Sigma no. H-3506) to facilitate the removal of cumulus cells.

Embryo storage, running the activation protocol and Ca^{2+} monitoring were all carried out in TCM 199 culture medium (GIBCO BRL, France) supplemented with 6 mg/ml of BSA. Inhibition of the extrusion of the 2nd polar body during the process of egg activation was obtained by adding 0.2 μg/ml of cytochalasin D (Sigma, no. C8273) to the culture medium. Experimental eggs were cultured in TCM 199+6% BSA medium supplemented with 10% FCS (Dutcher no. 40801; France). Electrical field pulses (EF) for Ca^{2+} stimulation were delivered in a non-ionic medium made of glucose (30 g/l; BDH Aristar, England) and 100 μM Ca^{2+} in water (Elgarstat, 18 Mohm.cm).

Oocyte collection

Sexually mature mixed breed female rabbits were superovulated with injections of follicle-stimulating hormone (FSH; Stimufol, Belgium) and human chorionic gonadotropin (hCG; Chorulon, Intervet, France). Females received a total of 2 mg FSH in five intra-muscular injections at 12 hours intervals: 0.250 mg, 0.250 mg, 0.650 mg, 0.650 mg and 0.650 mg. Twelve hours later, before mating with vasectomized or fertile males, 30 UI hCG injections were administered to the females intravenously. Twelve to 14 hours after mating, the does were slaughtered and the oocytes or fertilized eggs were recovered. For fertilized eggs, the mating was carried out half an hour later to offset the delay needed to prepare the oocytes for parthenogenetic activation.

Measurements of intracellular free Ca^{2+} concentrations

Intracellular Ca^{2+} was measured using the Ca^{2+} sensitive fura dextran dye (fura dextran, potassium salt, 10,000 M_r; Molecular Probes no. F-3029, Netherlands). The $[Ca^{2+}]_i$ was monitored by measuring the fluorescence from individual eggs loaded into a specially designed chamber placed on the stage of an inverted microscope (TMD Nikon). The excitation wavelength was altered from 350 nm to 380 nm using 10 nm bandpass interference filters. The emitted fluorescent light was measured through a 590 nm longpass filter. The intensity of light was recorded with a photometer (type 9924B; Thorn EMI) for 200 milliseconds at each wavelength and the background measured in the surrounding medium was subtracted before final calculations. Free Ca^{2+} concentration is expressed as the 350 nm/380 nm ratio of fluorescence. The data collection and processing were controlled by a computer using the Newcastle Photometrics system (Ver. 4.82, Newcastle upon Tyne, UK). Recordings are presented as fluorescence ratio against time.

Ca^{2+} stimulation procedures for egg activation

The apparatus and procedures used for Ca^{2+} stimulation were similar to those previously described in full detail for mouse oocytes (Ozil and Swann, 1995). Two slightly different chambers were designed to fulfil the specific requirements for the experiments. (i) For intracellular Ca^{2+} recording during electropermeabilisation in a small perfusion chamber, a suction pipette was used to hold single eggs between two small electrodes (0.2 mm high and 1 mm long) 0.42 mm apart. (ii) For oocyte activation studies, batches of oocytes were activated in another type of chamber with longer electrodes (15 mm), between which they were held by suction through a longitudinal slit in the bottom of the chamber. About 60 eggs can be inserted and tested at one time in such a chamber. During the experiments, the media temperature was maintained at 38°C by a thermostatically controlled heating device. On each side of the chambers two small tubes made it possible to inject either culture or glucose medium. The glucose medium was perfused at 80 ml/hour for 5 seconds and then at 150 ml/hour for 5 seconds. The conductance of the medium during the pulse was monitored with a Tektronix 7704A oscilloscope. This measurement indicated that the culture medium had been efficiently removed before EF pulse application. After each EF pulse, the culture medium was immediately re-perfused into the chamber. The use of a rapid solution exchange system facilitates the resealing of pores and the decrease in $[Ca^{2+}]_i$ after the EF pulse (Ozil and Swann, 1995). Ca^{2+} influx is brought about by the Ca^{2+} concentration in the pulsating medium. It is a constant and fixed value at 100 μM.

In the present study, we used an oscillating field of 10 kHz as it has been shown that radio frequency pulses cause less long-term damage to cells than the application of a simple bipolar pulse (Chang, 1989; Ozil and Swann, 1995). The pulse is composed of six 45-microsecond bipolar pulses at 5-microsecond intervals. The amplitude of the Ca^{2+} change was modulated by the amplitude of the voltage of the pulse

while the duration of the train was kept constant and fixed at 300 microseconds. A set of 3 voltage amplitudes were used, 55, 65 and 75 Volts, representing a field strength of 1.30, 1.55 and 1.80 kV/cm, respectively, in the chamber.

For all of the experiments the oocytes were treated and cultured in the presence of cytochalasin D for an overall period of 90 minutes and then transferred to a drug free culture medium. The activated oocytes, thus, did not form the 2nd polar body and retained a diploid set of maternal chromosomes.

Oocyte treatments

A logical series of three experiments was organised.

Experiment I: the effect of the amplitude and the number of Ca^{2+} impulses on the efficiency of oocyte activation

The first objective was to determine the optimal set of electroporation parameters causing a series of intracellular Ca^{2+} changes matching as closely as possible the amplitude and time course of the spontaneous $[Ca^{2+}]_i$ rises triggered by fertilization.

We submitted batches of oocytes to either 1, 2, 3 or 4 pulses, the voltage amplitudes of which were 55 to 65 and to 75 V. The interval between the pulses was fixed at 8 minutes, corresponding to an intermediate value between the minimum of 4 minutes previously used to activate rabbit oocytes (Ozil, 1990) and the maximum of 16 minutes recorded after fertilization in rabbits (Fissore and Robl, 1993). The treated oocytes were cultured and the rate of activation was given by the proportion of eggs that formed pronuclei. In parallel, five intracellular Ca^{2+} recordings of individual oocytes were performed for each treatment and the average of the $[Ca^{2+}]_i$ changes were computed and charted using SIGMAPlot software.

Experiment II: the effects of the amplitude, the number and the frequency of Ca^{2+} impulses on the rate of cell division

The second objective was to determine if the dynamics of the early cell cycle are affected by the amplitude, the frequency or the number of Ca^{2+} spikes.

In experiment IIA, oocytes were submitted to four pulses given at 8-minute intervals with a voltage amplitude of either 55 V or 65 V. Hereafter these treatments will be referred to as *4P-55V-8min* and *4P-65V-8min*.

In experiment IIB, oocytes were submitted to four pulses of 65 V given at either 16 minutes or 32 minutes (*4P-65V-16min* and *4P-65V-32min*).

In experiment IIC, oocytes were submitted to eight pulses of 65 V given at 4-minute or 8-minute intervals (*8P-65V-4min* and *8P-65V-8min*).

The time course of cleavage of the activated oocytes was monitored by counting the number of oocytes that cleaved in vitro at a fixed time, i.e. 12, 13, 14 and 24 hours after the onset of the treatment. After these periods of time, all 4- to 8-cell embryos were immediately transferred in groups of 8 into the oviducts of pseudopregnant does. As a control experiment, fertilized eggs were also transferred under similar conditions.

Experiment III: the effects of the amplitude, the number and the frequency of Ca^{2+} impulses on the postimplantation viability

The third objective was to evaluate the postimplantation viability at day 11.5 for each of the eight treatments described in experiment II. We chose this postimplantation stage because it represents the ultimate stage of parthenogenetic development in the rabbit species (Ozil, 1990). This stage is within the period of uterine enlargement which characterises the growth acceleration of the conceptus (Reynolds, 1947). There is a local uterine distension at each implantation site that is spheroidal and the size of which characterizes the growth performance of each category of treated eggs. Although the external dimensions of the site include a maternal component such as decidua basalis, endometre and myometre, its major proportion originates from the conceptus itself (Reynolds, 1947). Therefore, the simplest technique to estimate the developmental growth of the placenta before dissection was to measure the mesial diameter (MD) and the lateral diameter (LD). We estimated the volume of the implantation site by calculating the ovoid volume using the formula $V=4\pi/3.((MD/2.(LD/2)^2)$. The conceptuses were freed of the maternal and fetal membranes and the fetuses were collected.

Their gross anatomy was examined under a dissecting microscope. Each conceptus was classified into one of three classes according to the following morphological criteria. In class A, we grouped fetuses that displayed a general shape similar to that of fertilized embryos except that they were of smaller crown-rump size with no obvious anomalies (Fig. 1i, ii, iii). They had a good cephalocaudal flexion, which explains why we measured their crown-rump and head sizes. In class B, we recorded fetuses that showed a large range of anomalies, such as no proper cephalocaudal flexion (Fig. 1ii, iii, iv), that made measuring the crown-rump length meaningless. Sometimes fetuses had abnormally large heads and translucent brains with no folding of the posterior part of the body. The posterior part, in fact, remained underdeveloped (Fig. 1iv) in such cases. In class C, we recorded conceptus with no recognizable developmental organisation or showing severe developmental retardation (Fig. 1ii, iii, v). In some cases we recovered only the fetal envelope with no trace of fetal organisation (Fig. 1vi). In other cases the resorption process was too advanced to find any embryonic tissue.

Statistical analysis

Data from experiments II and III are presented using descriptive statistics with box plots. For statistical analysis, individual percentages for each recipient doe were used. The effect of the treatments was analyzed by one way variance analysis using arcsine transformed data. This method takes into account the variability among recipients. When a significant *F* ratio was found, pairwise comparisons were made using the Tukey test. Differences were considered as significant when $P<0.05$. Statistical analysis and box plots were made using Systat 7 software.

RESULTS

Experiment I: the effect of the amplitude and the numbers of Ca^{2+} impulses on the efficiency of oocyte activation

A graphical representation of the results is given in Fig. 2. The average $[Ca^{2+}]_i$ recorded for each treatment is associated with the rate of activation obtained after each pulse. Each $[Ca^{2+}]_i$ record represents an average of at least 5 different oocytes. The graphs make it possible to visualize the graded responsiveness of oocytes following repeated Ca^{2+} stimulation. They clearly show that the rate of egg activation is a complex function of the amplitude and the number of Ca^{2+} signals. For a single pulse of 55, 65 or 75 V the rate of activation is 5%, 14% and 13%, respectively. The rate increases progressively with the number of Ca^{2+} pulses and, after the fourth, reaches 53%, 95% and 100%, respectively.

Ninety-five percent of the oocytes responded after receiving 4 pulses of 65 V (*4P-65V-8min*), unlike oocytes submitted to higher amplitudes (i.e. *4P-75V-8min)*, which all responded to the treatment (100%). Such sub-optimal response indicates that the amplitude of Ca^{2+} change caused by 65 V in the activation chamber is not too massive, perhaps close to the value of spontaneous Ca^{2+} transient.

Fig. 3 shows two graphs of intracellular Ca^{2+} change. Fig.

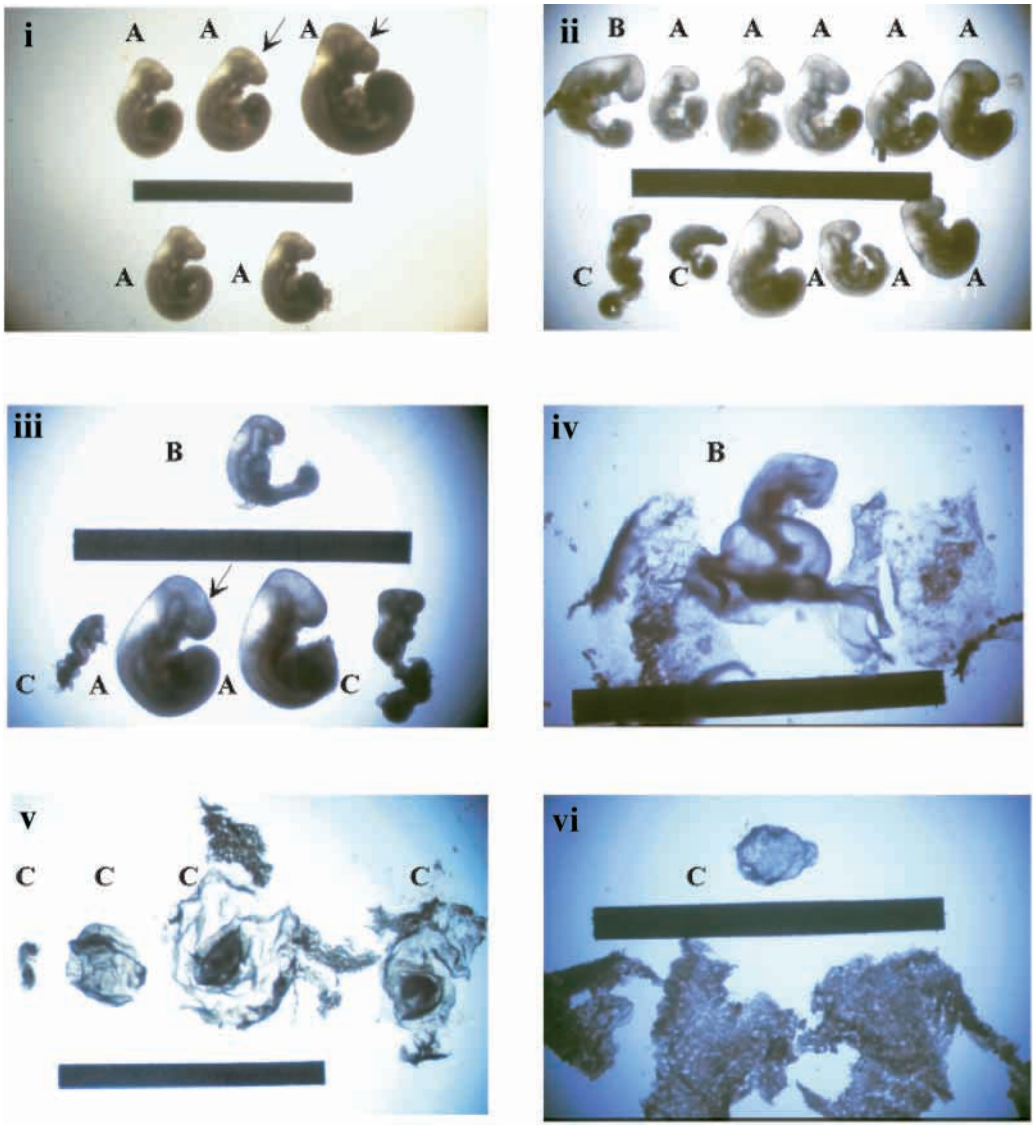

Fig. 1. Examples of the three classes of rabbit conceptuses at day 11.5 of pregnancy. (i) Conceptuses from fertilized eggs showing some degree of size heterogeneity but with typical morphological development. They display good cepahalocaudal torsion. The region overlying the forebrain vesicle and the midbrain vesicle are separated by a fissure, indicated by an arrow. They are recorded in class A. The scale is given by a small bar of platinum (10 mm × 1 mm) in the central position. (ii) Parthenogenetic conceptuses from diploid oocytes activated by treatment *4P-65V-8min*. This treatment produces conceptuses with varying morphological quality. Class A conceptuses are smaller than those from fertilized eggs, but their morphology matches the typical morphological development except that there is no trace of a fissure between the forebrain and the midbrain vesicles. Class B conceptuses display obvious alterations, such as abnormal cephalocaudal torsion. Class C conceptuses show embryoid bodies with severe developmental alterations. (iii) Parthenogenetic conceptuses from diploid oocytes activated by treatment *4P-75V-8min.* It can be seen that increasing the signal amplitude from 65 V to 75 V can still promote conceptuses with varying morphologies. However the crown-rump size of these A conceptuses was greater (4.5 mm to 4.9 mm). These two fetuses have very good shapes and there is some evidence for the fissure between the forebrain and the midbrain vesicles. (iv) A parthenogenetic conceptus from an oocyte activated by treatment *4P-65V 32min*. This is a dead embryo partially enclosed in a fetal membrane. The posterior part of the body is proportionally underdeveloped. The brain is large but translucent and the optic primordium has an exaggerated oblong shape in comparison to fertilized fetuses. This is an example of a class B fetus. (v) A series of parthenogenetic conceptuses activated by treatment *8P-65V-8min.* All are class C. It can be seen that the fetal envelopes are relatively abundant in comparison with the sizes of the embryos. (vi) A small conceptus from an oocyte activated by *4P-65V-32min.* This is a class C conceptus. Only the trophoblastic membrane and the amnion were recovered. The amnion has a spherical shape but it is not possible to distinguish any form of embryonic disk or neural plate inside. It seems that the presence of the fetal membrane in absence of an embryo itself is more likely to result from an abnormal developmental pathway as a result of the treatment than from precocious resorption of the embryo itself.

3A represents an average record of 3 spontaneous Ca^{2+} transients measured some time after fertilization. The transient duration was measured at 50% of the maximum $[Ca^{2+}]_i$ peak and is equal to 18 seconds. In Fig. 2B, we show the average Ca^{2+} changes caused by EF pulse for 55, 65 and 75 V amplitudes on freshly ovulated oocytes. While the peak amplitudes vary with the EF amplitude, the duration of the change, at half amplitude, varies from 12 to 14 seconds. From these observations, we chose 65 V as a reference parameter for the amplitude.

This set of results shows that a minimum step of 10 V increases between electrodes, representing 0.23 kV/cm in the chamber, cause a range of $[Ca^{2+}]_i$ changes that can be sensed by oocytes. Such sensitivity indicates the accuracy with which the signal should be controlled for interpreting the biological function of the Ca^{2+} signal dynamics.

Experiment II: the effects of the amplitude, the number and the frequency of Ca^{2+} impulses on the dynamics of cell division

Table 1 summarises the results of the experiments for which the rate of cell division was recorded in relation to the

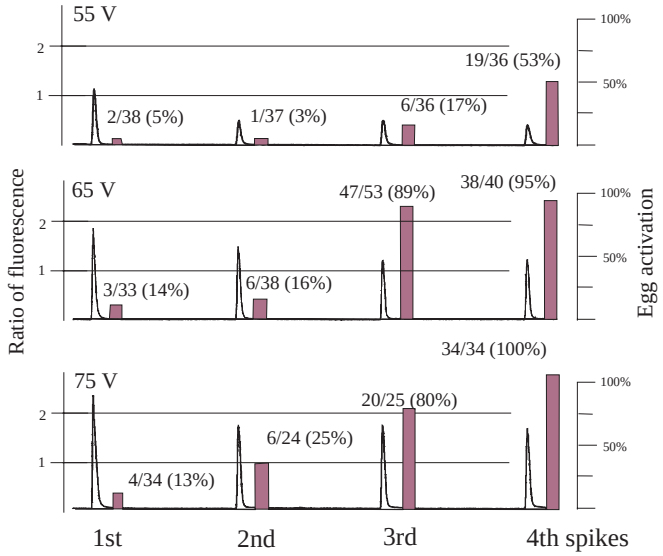

Fig. 2. Function of amplitude and number of Ca^{2+} spikes in driving oocyte activation. The graphs combine two types of records. Firstly, $[Ca^{2+}]_i$ change obtained in individual oocytes stimulated using three different EF parameters, 55 V, 65 V and 75 V, are plotted on the left side. Secondly, the corresponding rate of egg activation (as a percentage) obtained inside the stimulation chamber are plotted as bars on the right side of each signal. The time interval between calcium spikes is 8 minutes. This figure shows the oocyte's ability to respond gradually to small changes in amplitude of a repetitive signal.

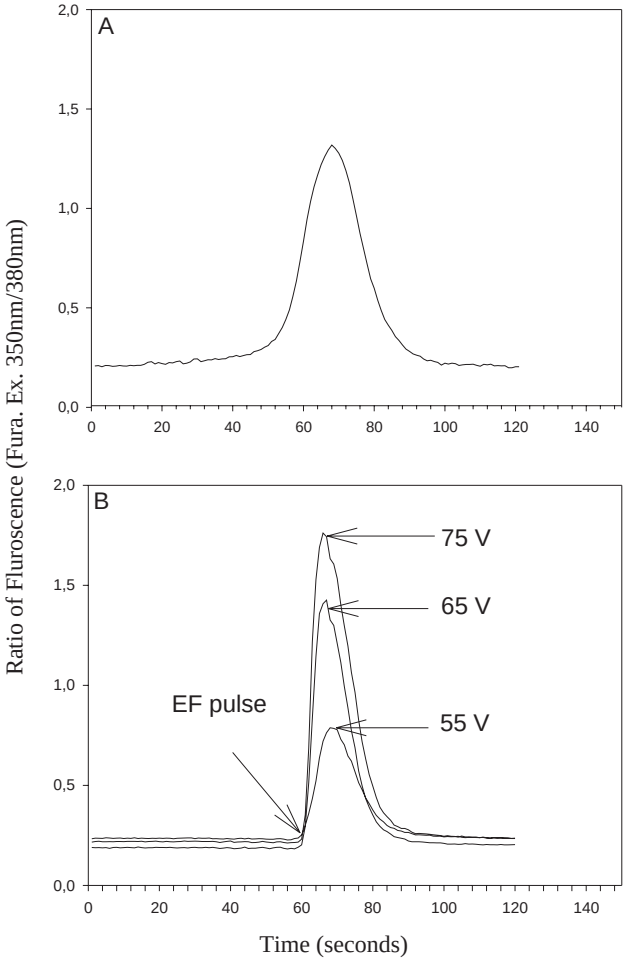

Fig. 3. Intracellular Ca^{2+} changes. (A) The average of three spontaneous Ca^{2+} transients recorded some time after fertilization and before the pronuclei formation on three different rabbit eggs using the measuring chamber. (B) The averages of $[Ca^{2+}]_i$ increase induced by EF pulse of varying amplitudes in non-fertilized rabbit oocytes stimulated in the measuring chamber. It can be seen that the down stroke is always very rapid. This could be due to rapid Ca^{2+} extrusion through the plasma membrane after EF pulsation. This characteristic makes the rabbit egg different from the mouse egg for which the time taken to restore the resting level of Ca^{2+} after EF pulsation is much longer.

amplitude, the frequency and the number of pulses. In the first series (Table 1A) two amplitudes of $[Ca^{2+}]_i$ change were tested (*4P-55V-8min* and *4P-65V-8min*). In the second series (Table 1B) two duration intervals between $[Ca^{2+}]_i$ changes were tested (*4P-65V-16min* and *4P-65V-32min*). In the third series (Table 1C), oocytes were submitted to eight $[Ca^{2+}]_i$ changes delivered at intervals of either 4 minutes or 8 minutes. The least efficient treatment was the one with the lowest EF amplitude (*4P-55V-8min*) which activates only 76% of the treated oocytes. In contrast, the five other treatments using a 65 V amplitude were all efficient since a total of 748 out of 764 treated oocytes became activated (98%). Fig. 4A,B show the cleavage status of activated and control eggs at 14 and 24 hours postactivation. The proportion of eggs that went from one cell to two cells at 14 hours postactivation was more variable for the treated eggs than for the controls (Fig. 4A). However, the median value of the percentages of two-cell eggs scored in the various treatments ranged from 76% (4P-55V-8min) to 90% (4P-65V-16min); the median was 88% for the fertilized controls. At 24 hours postactivation, (Fig. 4B) most of the eggs were at the four-cell stage or further. Overall, 98% of the activated eggs and 99% of the fertilized ones divided at least one time.

Experiment III: the effects of the amplitude, the number and the frequency of Ca^{2+} impulses on the postimplantation viability

Table 2 summarises the potential of treated eggs to implant and develop after transfer into pseudopregnant does. The proportion of recipients that became pregnant after receiving oocytes activated by *4P-55V-8min*, *4P-65V-8min*, *4P-65V-16min*, *4P-65V-32min*, *8P-65V-4min* and *8P-65V-8min* or fertilized eggs were not significantly different (χ-square test). The implantation rate of the eggs submitted to *4P-65V-8min* treatment (mean 57%) was similar to that of fertilized embryos controls, 52% per recipient doe. But when the pulse amplitude was decreased to 55 V, the implantation rate dropped to 9% and was significantly lower ($P<0.01$) than the 65 V treatment (experiment IIIA). In the subsequent experiments, 65 V pulses were delivered, varying the frequency and the number of pulses. The results were compared to those of the *4P-65V-8min* treatment. When the time interval was increased (experiment IIIB), the implantation rate decreased: with *4P-65V-16min* the mean implantation rate was 11%, compared with 57% ($P<0.05$). However, increasing the time interval to 32 minutes had less effect (28% versus 57%, $P=0.22$). Doubling the number of pulses with a time interval of 4 minutes or 8 minutes

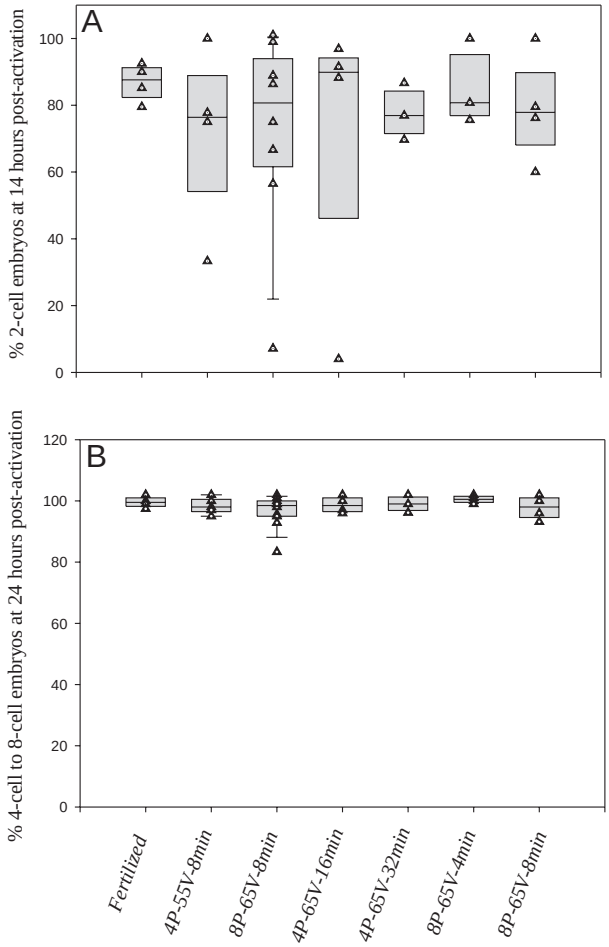

Fig. 4. Dynamics of egg division. (A) Box plot graph showing the proportion of embryos that went from one cell to two cells within 14 hours of the beginning of the treatment. The length of each box shows the range within which the central 50% of the values fell. The median value is represented by a transverse bar inside the box. (B) Box plot graph of the proportion of embryos that reached the 4- or 8-cell stage by 24 hours postactivation. No difference between treatments was found. The early cell dynamics does not appear to be influenced by the activation treatment.

(experiment IIIC) did not improve the implantation rate per recipient, which was 24% in both cases (P=0.04 and P=0.07, respectively).

If we consider the morphology and the size of the conceptuses, the long term impact of the treatment appears even more contrasted. When four Ca^{2+} signals were applied at 8 minute intervals but with a low amplitude (*4P-55V-8min*, see Fig. 2), all recovered conceptuses were very disorganised morphologically and were in resorption. In contrast, when the voltage was increased to 65 V (*4P-65V-8min*), causing a larger influx of Ca^{2+} ions inside the egg (see Figs 2, 3), the morphological aspect of the fetuses was much better (class A, see Fig. 1ii). From the 157 eggs transferred, 32 class A fetuses developed and the mean output per recipient was 20%. This rate of good fetuses (A) does not appear to be significantly lower than that recorded for the fertilized controls (35%; P=0.17). Such a proportion of well organized conceptuses was not obtained when the time interval between pulses increased to 16 or 32 minutes, since the recovery of class A embryos dropped to 3% (P<0.02) and 2% (P<0.01), respectively.

If we consider the overall production of fetuses, that is to say the cumulative number of classes A and B fetuses, we find that only the *4P-65V-8min* treatment was capable of promoting fetus development in a proportion similar to that of fertilized eggs, i.e. 42% vs 38% (see Fig. 5B). All the other treatments: *4P-65V-16min*, *4P-65V-32min*, *8P-65V-4min* and *8P-65V-8min* were less efficient. The mean percentages of fetuses were, respectively, 6% (P<0.02), 13% (P=0.06), 10% (P<0.02) and 8% (P<0.02). We did not find any difference in the occurrence of poorly developed conceptuses (C) between parthenogenetic treatments and the controls.

Table 1. Effects of the amplitude, the frequency and the number of Ca^{2+} pulses on the rate of cell division

Treatment	No. of oocytes	No. activated (%)	No. of 2 cell eggs (%) 12 h pa	13 h pa	14 h pa	No. of 4 cell 24 h pa	No. of 8 cell 24 h pa	Total cleaved
(a) Amplitude modulation								
4P-55V-8min	132	101	7/62*	27/62*	41/62*	89	12	101
		(76)	(11)	(44)	(66)	(88)	(12)	(100)
4P-65V-8min	212	202	58/173*	101/173*	139/173*	131/199*	63/199*	195/199*
		(95)	(34)	(58)	(80)	(66)	(32)	(98)
(b) Frequency modulation								
4P-65V-16min	157	155	46	103	120	115	37	154
		(99)	(31)	(66)	(77)	(74)	(24)	(99)
4P-65V-32min	99	95	25	48	72	78	10	88
		(96)	(28)	(54)	(78)	(82)	(11)	(93)
(c) Number modulation								
8P-65V-4min	167	167	55/121*	91/121*	106/121*	97	70	167
		(100)	(45)	(75)	(88)	(58)	(42)	(100)
8P-65V-8min	129	129	22	71	100	85	40	129
		(100)	(17)	(55)	(78)	(66)	(31)	(100)
Total of experimental eggs	896	849	213	441	578	595	232	834
		(95)	(29)	(60)	(79)	(70)	(27)	(98)
Fertilized control	166	126	48	90	109	79	46	125
		(76)	(38)	(71)	(87)	(63)	(36)	(99)

Hours postactivation (h pa) means hours after the first impulse.
*When records are not made on all the replicates, the quotient value is given.

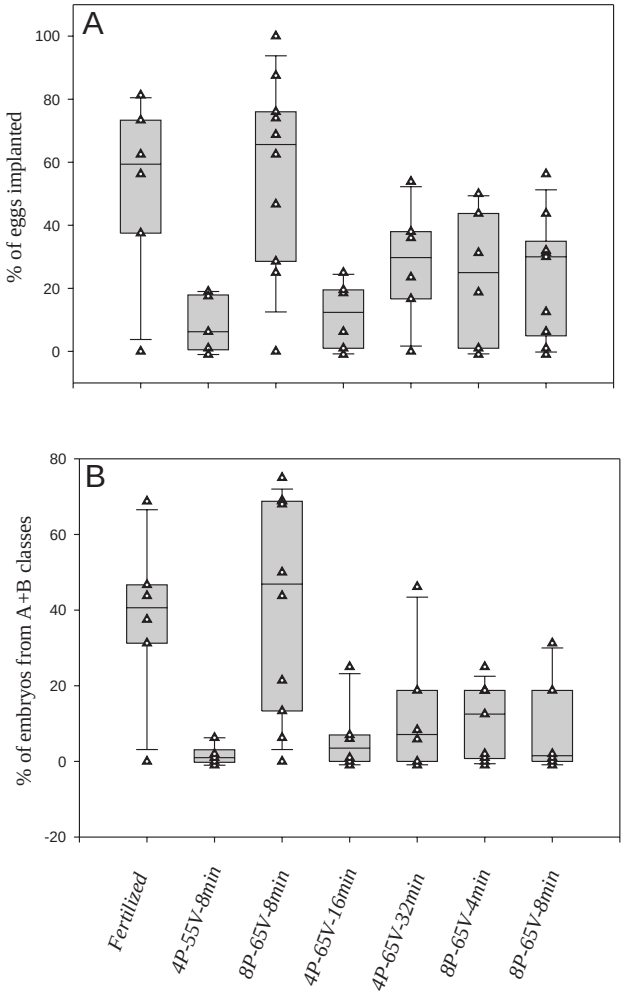

Fig. 5. Implantation of eggs. (A) A box plot graph showing the percentage of fertilized and parthenogenetic eggs implanted (ratio of the number of implantation sites to the number of oocytes transferred) for the individual recipients as well as the median value for each treatment. Only treatment *4P-65V-8min* gave similar implantation rates to those of fertilized eggs. (B) A box plot graph showing the percentage of the total number of eggs transferred that developed into embryos (A + B classes) for individual recipient does; the median value of these percentages for each treatment is also shown.

The class A parthenotes were recovered from placentas in which blood circulation was visible. Despite this fact, at the uterine junction of the antimesial upper part of the uterine enlargement we always noticed necrosis, the first sign of an inexorable process of abortion. Gross morphological examination revealed that A parthenotes reached the fore and hind limb bud stage and the optical pit stage, which normally occurs at day 11 (Leone, 1977). Also, there was heartbeat and blood circulation in a majority of the fetal bodies.

Table 3 summarizes the average sizes of the implantation sites, the sizes of the fetuses and their heads. The crown-rump size of A parthenotes varies from a maximum of 4.3 mm to a minimum of 2.5 mm, i.e. a range of 1.8 mm, with an average size of 3.2 mm representing 56% of the average size of fertilized fetuses at the same stage. In contrast, the average crown-rump size of the fertilized fetuses reached 5.7 mm with a maximum of 6.8 mm and a minimum of 4.3 mm, i.e. a range of 2.5 mm. In Fig. 6A, we plotted the volume of the implantation site versus the crown rump size of the class A parthenotes and fertilized fetuses. The graph clearly shows that the parthenotes are smaller than the controls but the placenta outgrowth appeared proportional to the size of the fetuses. These measurements clearly demonstrate that rabbit parthenogenetic conceptuses did not show a relative hypotrophy of the placenta as one would expect on the basis of previous experiments in the mouse (Surani et al., 1984). In order to detect unbalanced development, we plotted the head size in relation to their crown-rump sizes. Table 3 and Fig. 6B show that the relative head size of parthenogenetic class A fetuses tended to be slightly larger than that of fertilized fetuses. Moreover, it appears that parthenogenetic fetuses have no clear limit (fissure) between the forebrain and the midbrain (see Fig. 1). This absence of a fissure between the two brain vesicles appears to be an anatomical trait that characterizes the class A rabbit parthenotes. Among the two other classes, B and C, the range of anomalies was so diverse that we were not capable of finding a typical morphological syndrome. We noticed that sometimes no fetuses or very small fetuses were recovered from relatively large placentas, as shown in Fig. 1iv, but the reverse situation, that of a well developed fetus in a small placenta sac, was encountered in only one case, that of a fertilized embryo (see data on the plot Fig. 6A).

Table 2. Postimplantation development at day 11.5 in relation to the amplitude, frequency and the number of Ca^{2+} pulses

Treatment	No. of egg transferred	No. of recipient (average by doe)	No. of pregnant recipient (%)	No. of implantation (%)‡	Rate of implantation*,‡	No. of class A (%)§	No. of class A+B (%)§	No. of class C (%)§
(a) Amplitude modulation								
4P-55V-8min	80	5 (16.0)	3 (60)	7 (9)	15%	0 (0)	1 (1)	6 (8)
4P-65V-8min	157	10 (15.7)	9 (90)	90 (57)	63%	32 (20)	66 (42)	24 (15)
(b) Frequency modulation								
4P-65V-16min	96	6 (16.0)	4 (67)	11 (11)	17%	3 (3)	6 (6)	5 (5)
4P-65V-32min	87	6 (14.5)	5 (83)	25 (28)	34%	2 (2)	11 (13)	14 (15)
(c) Number modulation								
8P-65V-4min	144	9 (16.0)	7 (78)	34 (24)	30%	8 (6)	15 (10)	19 (13)
8P-65V-8min	96	6 (16.0)	4 (67)	23 (24)	26%	1 (1)	8 (8)	15 (16)
Fertilized eggs	95	6 (15.8)	5 (83)	49 (52)	62%	33 (35)	36 (38)	13 (14)

*The rate is obtained by considering only the number of embryos transferred to the pregnant does.
‡Mean of the percentages of eggs implanted per recipient doe.
§Mean of the percentages of fetuses per recipient to the number of eggs transferred.

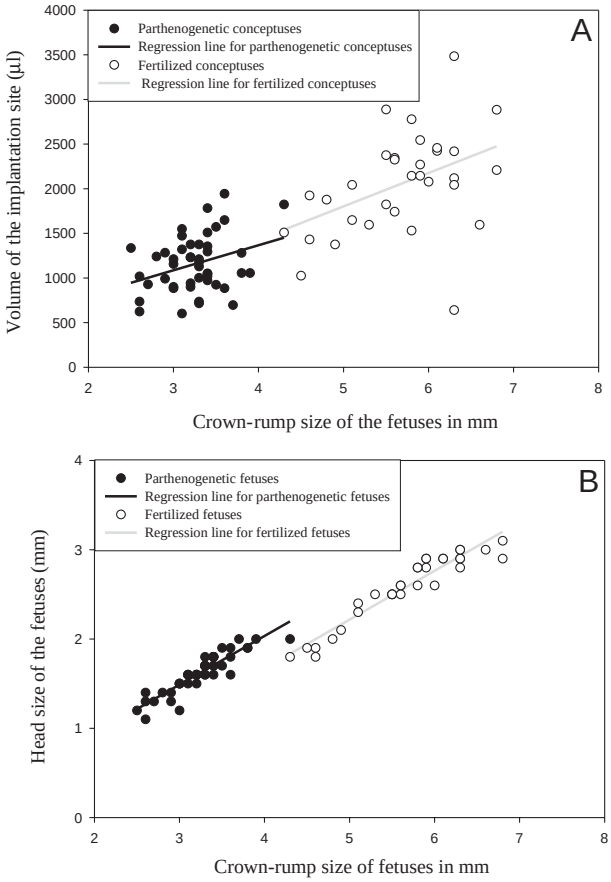

Fig. 6. The relative growth of rabbit parthenogenetic conceptuses. (A) The volume of the implantation site is plotted against the crown rump size of the A class parthenogenetic and fertilized rabbit fetuses at day 11.5 of pregnancy. The parthenotes are smaller than the fertilized control but the growth of the fetal envelope appears to be proportional to the fetus itself. In the control group we found one case of a large fetus in a small implantation site. (B) The relative development of the head size versus the crown-rump size. It appears that the parthenogenetic fetuses head size, while smaller, are relatively larger than the fertilized control fetuses.

DISCUSSION

It has been known for some time that Ca^{2+} is a powerful activating agent for oocytes (Steinhardt et al., 1974; Whittingham, 1980; Cuthbertson and Cobbold, 1985; Miyazaki et al., 1986; Ozil, 1990; Vitullo and Ozil, 1992). The present study was undertaken to determine whether or not the alterations of the regime of Ca^{2+} signal during activation impact on the developmental processes beyond the implantation stage. Recent trials to promote parthenogenetic development in large animals such as cattle (Fukui et al., 1992), sheep (Loi et al., 1998), and pig (Kure-bayashi et al., 2000) have shown that monotonic $[Ca^{2+}]_i$ changes triggered by either ionomycine or a single EF pulse are sufficient to promote some advanced postimplantation development. Such results underline the usefulness of the monotonic Ca^{2+} signal in causing egg activation in large animals but they do not provide much information about how postfertilization events, and especially the dynamics of the Ca^{2+} signal, exert a long-term influence.

In fact, the maximum developmental stage reached by parthenogenetic fetuses does not of itself constitute a good evaluation of Ca^{2+} signal efficiency because the parthenogenetic development is, above all, limited by a deficiency of expression of paternally derived imprinted genes (Surani et al., 1984; Solter, 1988). For this reason, it would be better to use fertilized eggs, which do not present genetic weakness. But precise manipulation of the changes in $[Ca^{2+}]_i$ in fertilized eggs remains uncertain due to its self-regenerative nature which is sperm-factor dependent (Swann, 1993; Faure et al., 1999). In addition, it has been shown that the technique used to control the spontaneous Ca^{2+} transients after fertilization might have some deleterious effects (Lawrence et al., 1998) that bias the long term effect.

For all these reasons, we decided to use parthenogenesis as a developmental assay since the absence of CICR sensitisation by fertilization makes it possible to bypass the natural processes and gain better control of the amplitude, number and frequency of the Ca^{2+} signal. The parthenogenetic development is sufficiently advanced in mammals after implantation to make it possible to assess the impact of the modulation of the Ca^{2+} signal on the many successive crucial transitions that an embryo has to go through to implant in the mother's uterus and to start growing and differentiating. The normal pregnancy duration for fertilized rabbit oocytes is 29-31 days (Reynolds, 1947) with implantation occurring at day 6 (Leone, 1977).

In this work we show that varying the value of the EF strength makes it possible to tune the amplitude of Ca^{2+} influx in such a way as to make the artificial signal higher than, lower than or similar to the natural Ca^{2+} released from internal stores. Hence, with the signal under tight experimental control, the technique offers the possibility of varying the number and the temporal sequence of the repetitive signal at will. Moreover, the physical nature of electroporation prevents any negative feedback mechanism at the cellular level that risks

Table 3. Sizes of the implantation site, crown-rump and head size of class A rabbit fetuses at day 11.5 of development

Treatment	No. of class A conceptus	Average mesial diameter (mm)	Average of lateral diameter (mm)	Average estimated volume (µl)	Average crown-rump size (mm)	Average head size (mm)	hs/crs ratio
4P-55V-8min	0						
4P-65V-8min	32	11.6	13.9	1197	3.2	1.6	51%
4P-65V-16min	3	11	14.7	1280	3.5	1.8	51%
4P-65V-32min	2	10.5	13	929	3.5	1.7	49%
8P-65V-4min	8	10.8	13	978	3.1	1.5	48%
8P-65V-8min	1	12.5	14.5	1376	3.2	1.6	52%
Total	46	11.4	13.8	1157	3.2	1.6	50%
Fertilized eggs	33	14.2	16.5	2046	5.7	2.6	45%

extinguishing the signal with time. The Ca^{2+} signal effect ceases when treatment is ended since Ca^{2+} ions per-se do not cause a regenerative response in the absence of fertilization (Igusa et al., 1983; Swann, 1994; Fissore and Robl, 1994; Ozil and Swann, 1995). It is thus possible to precisely target the period of time during which the later developmental effect originates. The modulation of parthenogenetic development obtained in the present study demonstrates the oocyte capability to sense Ca^{2+} very efficiently, whether the Ca^{2+} ions are released from internal stores by a second messenger or enter directly from outside by membrane electropermeabilization.

The results we describe here provide three major pieces of information. Firstly, Ca^{2+} stimulus is the most efficient signal activating mammalian eggs when applied in a repetitive manner, the amplitude of which is the crucial factor. Secondly, within the range of stimuli applied in the present study, the dynamics of early cleavage does not appear to be determined by either frequency or amplitude modulation of the Ca^{2+} signal that triggers cell cycle reinitiation. Thirdly, amplitude and temporal modulation of the Ca^{2+} signal during the early minutes affects the developmental performance and the morphology of the parthenogenetic conceptus at day 11.5 of pregnancy. These results are discussed below.

The effect of the amplitude of Ca^{2+} change

Within the range of parameters tested, the *4P-65V-8min* treatment gives the optimal developmental response (Table 2 and Fig. 5). It is noteworthy that in 6 out of 10 trials with the *4P-65V-8min* treatment, the rate of implantation was over 60%. This value corresponds to the median value reached by fertilized eggs. In contrast, none of the other 32 trial experiments reached such a level. Moreover, among the 9 recipients that became pregnant after receiving oocytes treated by the *4P-65V-8min* treatment, we found one recipient with 100% (16/16) implantation, a second recipient with 88% (14/16) and two with 75% (12/16) (Fig. 5B). Among these four pregnant recipients, a total of 54 out of 64 transplanted embryos (i.e. 84%) were able to successfully implant. While exceptional, such high rates of development show the precision of the developmental prediction that could be offered by a tight control of the intracellular Ca^{2+} dynamics during the early minutes of development.

However, the control is not absolute since the parthenogenetic embryos are subject to a large variety of disorders. Only the *4P-65V-8min* treatment produced a high proportion of high quality fetuses (32 out 90 i.e. 35% class A, see Fig. 1ii) and a relatively low proportion (24 out 90 i.e. 26%) of poor quality fetuses (class C, see Fig. 1ii and Table 2). Such variability appears to be integrally related to the way in which the oocyte decodes the signal. The only parameter which can differ from egg to egg is the amplitude of the $[Ca^{2+}]_i$ transients. The other parameters, such as the number and the time interval, are strictly identical. Such an amplitude effect is well illustrated in Fig. 2, but at this stage of the study we are unable to describe a causal relationship at the molecular level between the four Ca^{2+} impulses and the phenotype of the conceptus. In a few exploratory trials with *4P-75V-*8min (results not shown in detail) we obtained more advanced parthenogenetic fetuses (see Fig. 1iii). This graded phenotypical response suggests that a diploid genome of maternal origin is capable of promoting a relatively good placental development in the absence of any paternal contribution. Nevertheless, even the most advanced embryos are growth-retarded and their organogenesis appears to be unbalanced, since the head size of the parthenotes is larger than that of fertilized embryos (Fig. 6B). This is likely to be the consequence of the lack of genetic products coded by paternally expressed imprinted genes. However, the correlation between the phenotypes observed and the initial parameters of Ca^{2+} treatment used in activating the eggs suggests that the expression of imprinted and perhaps even of non-imprinted genes could be altered and might be responsible for the large spectrum of phenotypes. But how can simple non-specific events like variation in the amplitude or the frequency of Ca^{2+} oscillations during the period of egg activation influence the expression of genes 11 days later ? We hypothesize that the Ca^{2+} treatment, in addition to activating the oocyte to resume the cell cycle, can interfere with the epigenetic reprogramming of the zygote genome. These epigenetic changes (epimutations) are transmitted through the division of embryonic cells and the resulting altered gene expression pattern gives rise to morphological alterations that correlate with the initial treatment.

The effect of the time interval between Ca^{2+} signals

The time interval between the Ca^{2+} pulses also has some consequences on development (see Table 2) but the effect does not follow a linear progression. Increasing the time interval from 8 minutes to 16 minutes results in a decrease in the rate of implantation from 57% down to 11%, but when the time interval is further increased to 32 minutes, a slight re-increase in the rate of implantation, up to 26%, can be observed. However, since the age of the oocyte also increases, it is possible that the time dependent increase in the susceptibility of eggs to activation (Xu et al., 1997) interferes with the downstream action of the Ca^{2+} signal. The overall treatment lasts 128 minutes, meaning that the oocyte is naturally considerably more susceptible to activation in this treatment than in shorter ones. Nevertheless, it seems that there is an optimum length of time between Ca^{2+} changes that maximises the long-term response since simply doubling the time interval from 8 minutes to 16 minutes significantly decreases average survival probability. This result suggests that the efficiency of the enzymatic processes downstream from the Ca^{2+} signal is conditioned by periodical processes (Goodwin, 1965; Berridge and Galione, 1988; Goldbeter, 1996).

The effect of the number of Ca^{2+} signals

While fertilized rabbit eggs exhibit varying numbers of Ca^{2+} spikes (McCulloh et al., 1983; Fissore and Robl, 1993), it is surprising that only four Ca^{2+} rises are capable of efficiently promoting postimplantation development. It is interesting to note that doubling the dose of the total Ca^{2+} injected into the egg at 4 or 8 minute intervals is detrimental to the survival rate rather than beneficial. Eight pulses imposed in 32 minutes or 64 minutes appear significantly less efficient than 4 pulses imposed in 32 minutes. It thus appears that too intense a stimulation or excessive prolongation of high Ca^{2+} stimuli have a deleterious effect on the survival rate. Since fertilized rabbit eggs exhibit Ca^{2+} oscillations up to the pronuclear apposition (Fissore and Robl, 1993) but with a slow drop in amplitude to the point at which they are no longer generated due to a progressive degradation of the InsP3 receptors (Brind et al.,

2000; Jellerette et al., 2000), it is possible that prolongation of the Ca^{2+} regime beyond the first 4 impulses but with a progressive attenuation of the signal (Ozil, 1990) would standardise the developmental response even more.

Interpreting the developmental impact of the Ca^{2+} signal

Fluctuations in $[Ca^{2+}]_i$ are known to mediate a very large array of cell functions including chromosome motion (Izant, 1983; Zhang et al., 1990), cycle regulation (Hepler, 1989; Whitaker, 1997; Groigno and Whitaker, 1998), extensive changes in non-histone chromosomal proteins (Stros et al., 1994), chromatin configuration (Dobi and Agoston, 1998) and gene expression (Dolmetsch et al., 1997; Dolmetsch et al., 1998; Li et al., 1998).

Such nonspecificity of the Ca^{2+} signal and its influence on a large array of interconnected and synchronized functions complicates comprehension of the mechanism by which small variations in $[Ca^{2+}]_i$ or their temporal sequencing influence the developmental processes at later stages. Thus, in order to better separate the effects that might originate from abnormal zygotic genome expression from those that might accompany the activation process, such as abnormal distribution of chromosomes in the daughter cells or abnormal cell cycle reinitiation after the MII stage, we carried out precise observation of the cellular dynamics.

Does the Ca^{2+} regime during activation compromise the diploid status of the oocyte?

Artificial activation might cause abnormal distribution of chromatides and chromosomes in sister cells due, firstly, to the absence of male centrioles that normally participate in the organisation of the functional furrow at mitosis and, secondly, to the non-regulated influx of Ca^{2+} that is known to perturb the functioning of microtubules. In our study we found three main points that lead us to believe that both these problems can be ruled out. Firstly, the presence of cytochalasin during the treatment ensures the diploid status of the oocyte by preventing extrusion of the second polar body or any loss of individual chromosomes. The visualisation of two pronuclei in the center of each egg after treatment means that the range of Ca^{2+} stimulation was just sufficient to cause chromatide disjunction but not their dispersion, as can occur with excessive Ca^{2+} stimulation (Ozil, 1990). Secondly, since all of the embryos went from one cell to two cells at cleavage rather than forming three or four blastomeres, we can rule out any risk of formation of haploid or aneuploid mosaic embryos due to non-apposition of the two pronuclei (Tarkowski et al., 1970; Ozil and Modlinski, 1986; Schatten et al., 1991; Plachot and Crozet, 1992). Thirdly, it has been shown that rabbit oocytes have all the components for centriole assembly and are capable of forming centrioles spontaneously after appropriate stimulation (Szollosi and Ozil, 1991).

On the basis of these cellular observations, we assume that the abnormal distribution of chromosomes concomitant with the modulation of the Ca^{2+} signal does not seem severe enough to account for the degree of developmental restriction observed at postimplantation stages.

Does the Ca^{2+} regime during activation compromise the dynamics of the early cell cycle?

Ca^{2+} fluctuations are assumed to play a pivotal role in regulating the cell cycle (Poenie et al., 1985; Hepler, 1989). We therefore monitored the dynamics of early cleavage to reveal any correlation with the regime of $[Ca^{2+}]_i$ change. The results show that almost all activated eggs undergo early cleavage according to a normal schedule irrespective of the activation treatment (see Fig. 5). Since the process of CICR does not function in the absence of fertilization (Miyazaki et al., 1992; Kono et al., 1995) and electroporation of Ca^{2+} never causes spontaneous Ca^{2+} release (Fissore and Robl, 1993), we assume that the only Ca^{2+} changes affecting oocytes are those imposed during the treatment. Therefore, the biological effects associated with Ca^{2+} change cease after the final Ca^{2+} impulse. We can, hence, infer that the occurrence of fairly synchronous cell cycles might mean two things. Firstly, the sensitisation of CICR by the incoming sperm at fertilization does not appear to be the crucial event regulating the early cell cycle. It is possible that the resting level of $[Ca^{2+}]_i$ is sufficiently high to allow a correct transition between the cell cycle check points without the need of a spontaneous spike. Secondly, the cell cycle regularity in absence of regenerative Ca^{2+} release suggests the existence of a biochemical oscillator to pace the cell cycle independently of $[Ca^{2+}]_i$ oscillations, as previously suspected by Hepler (1989) and Roussel (2000). A good deal of data and several theoretical models propose that cell cycles are finely tuned by the Ca^{2+} signal (Swanson et al., 1997). Here we have produced results with rabbit embryos that give another picture. These findings strongly suggest that abnormal developmental patterns observed in the present study are not caused by a differential rate of early cell division.

Conclusions

This experiment clearly reveals a hidden behaviour of mammalian eggs in response to oocyte activation. That is to say, small changes in the Ca^{2+} signal dynamic have a strong impact on both the immediate oocyte response (activation) and the long-term postimplantation development (phenotype) while at the intermediate stages the cell division dynamics appears to be less affected. This time gap before reaching the full developmental response makes it difficult to understand how the Ca^{2+} dynamics influence development. This difficulty seems to reinforce a general idea that once oocytes have been activated, the dynamics of egg activation no longer exercise a strong impact on the developmental process. We have shown here that the activation process participates in the determination of the developmental processes. It remains to be seen how. It is known that $[Ca^{2+}]_i$ oscillation directly influences the expression of numerous developmentally regulated genes in cultured cells (Fields et al., 1993; Sheng et al., 1993; Dolmetsch et al., 1997; Dolmetsch et al., 1998; Li et al., 1998). It is, however, unlikely that the mechanisms of action inside the oocyte resemble those affecting such gene expression since the impact ceases at the last Ca^{2+} impulse, while the chromosomes are still compacted and transcriptionally inactive. The fact that the effect endures suggests that the changes are inherited through DNA synthesis and cell division. It is quite possible that remodelling the chromosomes and chromatin structure by the Ca^{2+} regime contributes significantly to determining the functional pattern of gene activity at later stages (Thompson et al., 1995; Schultz et al., 1999; Wolffe and Guschin, 2000). Of course the molecular basis for this sensitivity remains to be explained but the

existence of an optimal regime of Ca^{2+} stimuli with a temporal sequence demonstrates, firstly, the importance of epigenetic events during the postfertilization period and, secondly, the efficiency of Ca^{2+} signal modulation in studying the postfertilization events. Once a better understanding of how oocytes integrate signals from outside is available, the possibility of maximising the developmental response by proper Ca^{2+} stimulation after in vitro fertilization or intracytoplasmic sperm injection (Yanagida et al., 1999) could considerably increase the efficiency of in vitro techniques used in reproductive medicine.

We thank Andras Paldi and Yves Maurin for stimulating discussion. We are grateful to Aline Solari for help with the statistical tests and to Jean-Paul Renard for critically reading the manuscript. We appreciate the assistance of Donald White for the English revision of the manuscript.

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