The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation

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Summary
Freshly ovulated rabbit oocytes were activated parthenogenetically by periodically repeated calcium stimuli generated by electric field pulses applied onto the plasma membrane. Electric field pulses of 1.8 kV cm⁻¹ were delivered every 4 min for 1 h 30 min (22 double pulses) in a specially designed chamber. Before each pulse, the culture medium was replaced by an isotonic glucose solution containing 10 μM Ca²⁺.

The effects of modulating the ionic stimuli (by changing the duration of EF pulse) on a postactivation reaction, and/or on the pre- and postimplantation development, were studied.

The rate of activation increased progressively as the pulse duration lengthened. For 22 pulses of 200 μs, 13% of oocytes were activated versus 100% for 1200 μs. The uniformity of the parthenogenetic response was obtained when oocytes were exposed to a series of pulses within which the reduction of pulse duration followed a negative exponential law. The influence of such activating treatment on the preimplantation development was tested using two treatments of 22 pulses with a total pulse duration equal to 14868 and 11228 μs, respectively. For the weaker treatment, a lower proportion of embryos underwent compaction and those that compacted were irregular. In contrast, the majority of embryos resulting from the stronger treatment compacted and developed into blastocysts. The most significant result that emerges from this study is that the level of stimulation affects in vitro developmental potency after the third cleavage division.

The postimplantation viability of parthenogenetic eggs was tested and the results showed that parthenogenetic rabbit embryos died at a similar stage of development to the parthenogenetic mouse embryos. But, in the present series, high implantation rates and embryonic development (66%) till day 10–11 of pregnancy were obtained after the appropriate pulsatile EF treatment of oocytes. The parthenogenetic fetuses were of smaller size than the controls, but the development of the trophoblast tissue was proportional to the development of the fetuses. Anomalies of fetuses were also observed. This study reveals that activation is not a time-limited event and that the type of activating treatment has a marked effect on the ability of the resulting parthenogenetic embryos to develop to the early postimplantation stages.

The sustained alteration of the cytoplasmic activity provides a useful tool to study the function of embryonic or somatic nuclei introduced during the earliest stages of activation.

Key words: parthenogenesis, repetitive electrical stimulations, rabbit, oocyte, long-term effect.

Introduction
In mammalian eggs, fertilization is accompanied by a transient increase in the intracellular free-calcium ion concentration ([Ca²⁺]) followed by a series of periodic Ca²⁺ waves due to release of Ca²⁺ from the intracellular stores (Cuthbertson et al. 1981; Cuthbertson and Cobbold, 1985; Miyazaki et al. 1986).

The subsequent oscillations of intracellular free calcium occur in the whole egg and recur at fairly regular intervals for at least four hours following fertilization (Cuthbertson and Cobbold, 1985; Miyazaki et al. 1986). The means by which sperm–egg fusion triggers a sequential intracellular Ca²⁺ release remains unknown, but a signal transduction involving activation of a GTP-binding protein and IP3 and/or Ca²⁺-induced Ca²⁺ release is considered to be responsible for the repeated Ca²⁺ transients. (Miyazaki, 1988; Swann et al. 1989).

The biological functions of these long-lasting transient calcium oscillations are still unknown, but appear to be characteristic of fertilized eggs. This phenomenon was never observed when oocytes were artificially activated (Cuthbertson et al. 1981; Cuthbertson and Cobbold, 1985; Miyazaki, 1988). It is of interest to reproduce experimentally such oscillatory activity in the absence of the male gamete, i.e on the parthenogenetically activated oocytes, in order to determine the developmental events controlled or modulated by such sustained cytoplasmic signals normally induced after sperm–egg fusion.

It is well known that most mammalian eggs can be artificially activated by a variety of physical and chemi-
cal stimuli i.e thermal, electric or osmotic shocks, enzymes and anaesthetic agents (see review articles of Graham, 1974; Tarkowski, 1975; Whittingham, 1980; Kaufman, 1983). However, in spite of the variety of stimuli used, the activation treatment usually corresponds to a single time-limited event which is assumed to mimic the initial entry of the sperm into the egg. None of the above-mentioned artificial treatments are able to reproduce the series of rapid physiological changes occurring in the egg after sperm penetration. In many instances, the efficiency of activation with the various artificial stimuli depends on the age of oocytes (Kaufman, 1983).

In this study, attempts were made to sustain the release of calcium from internal stores by periodically repeated calcium stimuli generated by electric field pulses applied onto the plasma membrane (Zimmerman, 1982). It was assumed that calcium stimulation occurred as a direct consequence of the electroporation of the cell membrane. Earlier, in preliminary experiments, it was shown that the repeated application of such an external stimulus at a given frequency triggered the parthenogenetic activation of rabbit oocytes (Ozil and Stinnakre, 1988). Artificial activation of rabbit oocytes has not been as extensively studied as in the mouse, although it has been shown previously that cold shock (Pincus and Shapiro, 1940; Thibault, 1948; Chang, 1954) and electrical stimulation (Stice and Robl, 1988; Onodera and Tsunoda, 1989) can activate them. However, the response of rabbit oocytes to such stimuli and the developmental potential of the parthenotes were variable (Stice and Robl, 1988; Onodera and Tsunoda, 1989).

In this paper, a method for repeated ionic stimulation of ovulated rabbit oocytes is described as well as the effects of modulating the ionic stimuli (by changing the duration of electric-field pulse) on the pathways of activation and embryonic development.

**Materials and methods**

**Oocyte collection**

Sexually mature mixed-bred rabbit females were superovulated with subcutaneous injections of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) according to the technique described by Kennelly and Foote (1965) and modified by Thibault (personal communication). Females received 2 mg FSH in five injections at 12 h intervals: 0.250, 0.250, 0.650, 0.650 and 0.250 mg. Twelve hours later, prior to mating with a vasectomized male, they were injected with 0.33 mg LH.

Oocytes were recovered from the oviducts 12-15 h after mating by flushing in phosphate-buffered saline (PBS). They were incubated for 5 min in hyaluronidase (300 i.u. ml⁻¹ in PBS) to remove the follicular cells. After treatment, oocytes were cultured at 38°C in B2 medium (Ménézo, 1976) in a 5% CO₂ atmosphere.

**Method and experimental procedure**

The membrane permeability of freshly ovulated rabbit oocytes was transiently increased by opening pores with electric field pulse EF in the presence of 10 μM of Ca²⁺ contained in 0.3 M glucose solution – 18 MOhm H₂O (pulsating medium). It is assumed that while pores are open ions flow along their concentration gradients through the cell membrane into the cytosol, as shown previously in sea urchin oocytes (Rossignol et al., 1983). Thus, the ionic influx may be adjusted under these conditions by either the differential ionic concentration outside and inside or by the duration of the pulses.

The experimental procedure for the electrically induced ionic influx was similar to that previously described for electric field fusion of 2-cell rabbit embryos (Ozil and Modlinski, 1986). The oocytes were cultured with medium M16 (Whittingham, 1971) at 38°C in a specially designed chamber. Before each pulse, the culture medium was automatically replaced by the pulsating medium. Details of the chamber are described in Fig. 1. Each pulse consisted of two alternating pulses to prevent 'lateral electrophoresis' (Jaffe, 1977) of membrane proteins, which might occur after several pulses of the same polarity (Poo, 1981). The amplitude of the ionic signal was mediated through the duration of the pulse. The whole process was controlled by a microcomputer via a Tektronix MI 5010 interface with an MS-BASIC programme. The actual voltage and current between
electrodes were measured with a Tektronix 7704A oscilloscope mounted with a 7D20 programmable digitizer and a 7A22 differential amplifier.

The rhythm of electrical pulsation and the total duration of the treatment were the same for all treatments, i.e. applying one EF pulse every 4 min for 90 min. These values were chosen because they fitted well with the average frequency and duration of hyperpolarisation of the membrane potential of rabbit oocytes during fertilization (22 diphasic membrane potential oscillations during the first 90 min following sperm-egg fusion, 1 pulse every 4 min. McCulloh et al. 1983). The membrane potential variation is known to reflect the Ca\(^{2+}\) transient based on Ca\(^{2+}\)-activated K\(^+\) channels and is thus a reliable indicator of [Ca\(^{2+}\)] (Miyazaki and Igusa, 1982). The electric field amplitude (1.8 kV cm\(^{-1}\)) was constant for all experimental groups.

**Oocyte treatment**

The effects of different treatment parameters on oocyte activation was studied in four experimental groups.

**Group A. Experimental environment (controls)**

In order to test the effect of the experimental conditions (continuous perfusion, removal of culture medium and electric field pulses), freshly ovulated oocytes and fertilized eggs were given 22 double pulses of 900 \(\mu\)s in the pulsating medium without Ca\(^{2+}\) (first EF pulse, 13 to 15 h post-mating). After treatment, the fertilized eggs were transferred to pseudopregnant recipients to determine survival to term. Unfertilized oocytes were cultured in vitro and the rate of parthenogenetic activation recorded.

**Group B. Calcium ions and duration of EF pulses**

The effect of pulse duration was examined in the pulsating medium containing 10 \(\mu\)M CaCl\(_2\). Treatment in which oocytes were not activated was considered as minimum duration and that resulting in oocyte lysis as maximum duration. A set of six treatments with 22 constant double pulses was chosen arbitrarily. These treatments had a pulse duration equal to 200, 300, 600, 900, 1200 and 1500 \(\mu\)s, respectively. The effect of the presence of Mg\(^{2+}\) and Na\(^{+}\) ions at a concentration of 10 \(\mu\)M in the pulsating medium was tested with 22 constant double pulses with a duration of 900 \(\mu\)s.

**Group C. EF pulses modulation and the type of parthenogenetic activation**

The treatment with 22 double pulses of constant duration revealed the pulse duration values for which the maximum and minimum effects were recorded. These constant treatments did not produce a high rate of activation with a uniform type of parthenogenetic egg. In order to check whether a progressive reduction of the calcium stimuli in a given treatment may have any effect on the type of parthenogenetic reaction, oocytes were submitted to treatments in which the pulse duration decreased step by step according to a negative exponential relationship. Four treatments were tested according to the maximum duration of the first pulse. Fig. 2 gives the graph of the pulse durations for these treatments.

**Group D. EF modulation and development in vitro**

Does the strength of the activating treatment influence the preimplantation development? Such influence is best tested on diploid eggs since it is known, at least in mice, that very few haploid eggs develop to the blastocyst stage compared to diploid parthenotes (Tarkowski and Rossant, 1976). To avoid the potential influence of the effect of ploidy, oocytes were treated in the presence of 8 \(\mu\)g ml\(^{-1}\) cytochalasin B in the culture medium to block a second polar body extrusion and to obtain a uniform population of diploid parthenogenetic eggs. The influence of the strength of the treatment was tested using two treatments applied on group C: treatment I and treatment III within which the reduction of pulse duration followed a negative exponential law. Treatment I was the weak treatment with a total pulse duration of 11228 \(\mu\)s and treatment III the strong one with 14 808 \(\mu\)s. Parthenogenetic eggs were cultured in vitro to the blastocyst stage and the influence of the two treatments was evaluated by the rate of blastocyst formation.

**Postimplantation viability of parthenogenetic diploid embryos**

The postimplantation viability of parthenogenetic diploid embryos obtained with treatment III was tested. The embryos at the 4-cell stage were transferred to the oviduct of pseudopregnant does. Fertilized eggs were also transferred to the opposite horn in some recipients in order to compare the parthenogenetic development with the normal one. The recipients were autopsied between days 8.5 and 13 of pregnancy and the number of implantation sites and live fetuses were recorded.

**Results**

**Group A. Effect of experimental environment (controls)**

When the pulsating medium did not contain any electrolytes, none of the oocytes (105) given 22 double pulses activated. The experimental environment and culture conditions, i.e. the replacement of the culture medium before each pulse by a non-electrolytic pulsat-
ing media and the relatively strong electric field pulse treatment (2×1.8 kV cm⁻¹ × 900 μs × 22 times i.e. a total duration of pulses 39 600 μs) had no visible effect on freshly ovulated oocytes. In contrast, 41% (9/22) of the duration of pulses 39 600 μs had no visible effect on treatment (2×1.8 kV cm⁻¹ × 900 μs × 22 times i.e. a total duration of pulses 39 600 μs). It was not possible to totally replace the culture medium by the pulsating medium before each pulse was applied. The current measured during the pulse revealed that the conductivity of the pulsating medium was at least 15% higher than that measured between electrodes before the experiment. During the experiment, some ions from the culture medium were still present during the pulses and this modified the ionic generation of the signal. This signal depended also on the microenvironment under the zona pellucida which contained an unknown concentration of different ions. However, such microenvironments did not appear to have any significant effect on activation or embryonic development. In that series of experiments, it took 45 s every 4 min to wash the oocytes with the pulsating medium. The period during which the eggs were not in the culture medium did not have any significant effect on activation or survival to term.

**Group B. Calcium ions and EF pulse duration**

Table 1 summarizes the results of experiments in which the rate of activation was tested in relation to the pulse duration and in the presence of 10 μM CaCl₂. Parthenogenetic activation was recorded by the appearance of pronuclei after 3 to 4 h of culture. With a 200 μs pulse, only 6 out of 47 oocytes treated were activated (13%). The rate of activation rose progressively with increasing pulse duration. For 300 μs, 75 out of 99 oocytes were activated (76%); for 600 μs, 83 out of 85 (98%); for 900 μs, 63 out of 63 (100%); for 1200 μs, 92 out of 97 were activated and 5 destroyed by the treatment, for 1500 μs, 47 out of 50 were activated and 3 were destroyed. The results clearly show that the parthenogenetic activation was triggered when the pulsating medium contained Ca²⁺ (10 μM). Moreover, the rate of activation was directly related to the pulse duration which in turn was controlled by the extent of the calcium stimulation.

Table 1. Effect of calcium ions and EF pulse duration on the activation of rabbit oocytes

<table>
<thead>
<tr>
<th>Pulse duration* (μs)</th>
<th>No. oocytes</th>
<th>No. lysed (%)</th>
<th>% activated</th>
<th>1PN+PB1 &amp;PB2</th>
<th>2PN+PB1</th>
<th>&gt;2PN+PB1†</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μs</td>
<td>47</td>
<td>0 (0)</td>
<td>13</td>
<td>83</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>300 μs</td>
<td>99</td>
<td>0 (0)</td>
<td>58</td>
<td>68</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>600 μs</td>
<td>85</td>
<td>0 (0)</td>
<td>98</td>
<td>71</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>900 μs</td>
<td>63</td>
<td>0 (0)</td>
<td>100</td>
<td>62</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>1200 μs</td>
<td>97</td>
<td>5 (5)</td>
<td>100</td>
<td>57</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>1500 μs</td>
<td>50</td>
<td>3 (6)</td>
<td>100</td>
<td>30</td>
<td>17</td>
<td>53</td>
</tr>
</tbody>
</table>

* 22 pulses given in each treatment.
† abnormal pronuclei, small micronuclei.

Three types of parthenogenetic oocytes were produced after activation i.e. according to the nuclear configuration:-

1. One pronucleus (PN) plus the first and second polar bodies (PB1 and PB2) - haploid.
2. Two pronuclei plus the first polar body - diploid.
3. Several micronuclei (probably originating from disrupted metaphase or anaphase chromosomes) plus the first polar body - diploid, but abnormal.

As shown in Table 1, the nuclear configuration of parthenogenetic oocytes varied in relation to the treatment potential. The proportion of parthenogenetic eggs with 1PN and 2PB (Fig. 3A, B) decreased with increasing pulse duration. During the same time, the proportion of eggs with 2PN and those with micronuclei increased (Fig. 3C). These results show that the strength of ionic stimuli influenced not only the rate of activation, but also the nuclear configuration of parthenogenetically activated oocytes of similar postovulatory age. The longer the pulse duration, the larger the proportion of activated eggs, but the greater the proportion of oocytes containing micronuclei.

**Group C. EF pulse modulation and type of parthenogenetic activation**

Among the four treatments in which a progressive reduction of pulse duration was tested, the rate of parthenogenetic activation was 88% (76/86), 99% (150/151), 100% (118/118) and 100% (96/96), respectively (Table 2). The relationship between the type of parthenogenetic activation and each treatment is also shown in Table 2. Most parthenogenetic eggs had 1PN and 2PB. This proportion was 83% (63/76), 89% (134/150), 91% (107/118) and 70% (67/96), respectively, for treatments I to IV. The proportion of eggs with 2PN was about 9% in all treatments except treatment IV which was 25% (24/96). The results show that as the pulse duration was reduced, all oocytes were activated and the majority (91%) of them had a single pronucleus with 2PB when meiosis was completed (Fig. 3A, B).

Thus, the modulation of calcium stimuli through a variation of pulse duration seemed to be effective and influenced the first events of development. In this study, the age of oocyte was similar for all treated oocytes and thus the effect of ageing can be excluded from consideration.
Group D. Effect of EF modulation on the in vitro development of the parthenogenetically activated oocytes

When oocytes receiving treatments I and III (see Group C) were cultured in the presence of cytochalasin B, 92 (90/98) and 100% (352/352) of oocytes, respectively (Table 3), were activated and all of them contained two pronuclei when meiosis was completed (Fig. 4A). Of 69 oocytes subjected to treatment I (total pulse duration 11 228 μs) and cultured 5 days, only 23 (33%) reached the blastocyst stage (Fig. 4A,B,C,D). Of 244 oocytes subjected to treatment III (total pulse duration 14 868 μs), 89% (216/244) reached the blastocyst stage after 5 days in culture (Fig. 4A,B,E,F). No visible differences were recorded in the developmental potential of each group until the 3rd cleavage division (Fig. 4A,B). In parthenogenetic embryos produced from treatment I, a lower proportion of embryos underwent compaction and those that compacted were irregular (Fig. 4C,D). In contrast, the majority of embryos resulting from treatment III compacted and developed into blastocysts (Fig. 4E,F). These results show that the type of activating stimulus had a profound effect on the ability of the resulting parthenogenetic embryos to develop to the blastocyst stage in vitro.

Postimplantation viability of parthenogenetic diploid eggs

Thirteen recipients that became pregnant were autopsied between days 8.5 and 13 of pregnancy. 50 implantations were obtained from 165 (30.3%) transferred parthenogenetic embryos. No living embryos were collected after day 12 of pregnancy, but till day 11, 18 out of 27 collected embryos (66%) were alive (see Table 4). Although they were smaller than the controls, they appeared morphologically normal according to the criteria defined for the rabbit by Edwards (1968). The ratio between the external size of the implantation site and the crown-rump size of the fetuses was roughly equivalent for fertilized and parthenogenetic embryos (see Figs 5 and 6). Thus, it seemed that the general growth of parthenogenetic embryos, the fetuses with their trophoblastic tissue, was delayed. The dead

Table 2. Effect of EF modulation on pathway of parthenogenetic activation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. oocytes</th>
<th>% activation</th>
<th>1PN+PB1 &amp; PB2</th>
<th>2PN+PB1</th>
<th>&gt;2PN+PB1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>86</td>
<td>88</td>
<td>83</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>II</td>
<td>151</td>
<td>99</td>
<td>89</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>118</td>
<td>100</td>
<td>91</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>96</td>
<td>100</td>
<td>70</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

*abnormal pronuclei, small micronuclei.
Fig. 4. (A) Oocyte subjected to treatments I and III (group D) in the presence of cytochalasine B. All of them contained two pronuclei (magnification 110x). (B) Diploid parthenogenetic embryos at the 4- to 8-cell stage, 24 h postactivation. At this stage, no differences were visible between embryos treated by activating treatment I or III (group C) (magnification 60x). (C and D) Parthenogenetic embryos produced from treatment I (group D). The compaction of morula is irregular and only a few of them developed to blastocysts (magnification 60x). (E and F) Parthenogenetic embryos produced from treatment III (group D). The majority of them compacted and developed into blastocysts (magnification 110x for E and 60x for F).
Table 3. Effect of EF modulation on the subsequent development of diploid parthenogenetic eggs in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. oocytes</th>
<th>% activation</th>
<th>No. cultured</th>
<th>No. compacted morulae (%)</th>
<th>No. blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>98</td>
<td>92</td>
<td>69</td>
<td>25 (36)</td>
<td>23 (33)</td>
</tr>
<tr>
<td>III</td>
<td>352</td>
<td>100</td>
<td>244</td>
<td>244 (100)</td>
<td>216 (89)</td>
</tr>
</tbody>
</table>

Fig. 5. (A) A pregnant doe tractus on day 10.5 of pregnancy. 8 parthenogenetic diploid embryos were transferred into the left horn and 8 fertilized embryos into the right horn. 7 implantations of parthenogenetic embryos were obtained and they were all smaller than the 2 implantations of fertilized embryos in the contra lateral horn (one resorption of fertilized embryo is visible). The scale is given by a small bar of platinum (10 mm x 1 mm) in the upper part of the photo. (B) The 7 parthenogenetics fetuses are lying on the left side and only three of them are living with a beating heart (the first three on the left). The biggest is dead, but it is possible to see the blood storage in the heart. The others are abnormal and two do not even have a shape that resembles a fetus. The scale is given by the platinum bar (10 mm x 1 mm). Comparison of the external size of the fetuses and the external size of the implantation site shows that the development of the external envelopes is proportional to the size of the fetus or vice versa.

Table 4. Postimplantation development of parthenogenetic eggs submitted to treatment III

<table>
<thead>
<tr>
<th>Day of autopsy</th>
<th>D 8–9</th>
<th>D 9–10</th>
<th>D 10–11</th>
<th>D 12–13</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. eggs transferred</td>
<td>21</td>
<td>26</td>
<td>50</td>
<td>68</td>
<td>165</td>
</tr>
<tr>
<td>No. of implantations</td>
<td>9</td>
<td>3</td>
<td>15</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>(%) cumul</td>
<td>(42.8)</td>
<td>(21.2)</td>
<td>(27.8)</td>
<td>(30.3)</td>
<td></td>
</tr>
<tr>
<td>No. of living fetuses</td>
<td>8</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>(%) cumul</td>
<td>(88.8)</td>
<td>(91.6)</td>
<td>(96.6)</td>
<td>(96.6)</td>
<td></td>
</tr>
</tbody>
</table>
fetuses had several anomalies and it was not possible to classify them. The photographies show the diversity of those anomalies.

**Discussion**

In mammals, nuclear transplantation studies have shown that interactions between the male and female gametes after fertilization and during the first cell cycle are crucial for the development of new individuals. The paternal and maternal genomes have differential and complementary roles during embryogenesis and both are required for full development to term (Surani and Barton, 1983; McGrath and Solter, 1984; Surani et al. 1984; Mann and Lovell-Badge, 1986).

In the mouse, parthenotes appear to progress normally through preimplantation stages of development and to implant, but shortly after implantation they begin to degenerate and none survive beyond day 11 of gestation (Kaufman et al. 1977; Surani et al. 1986). It was demonstrated that parthenogenetic inviability is not only determined by two maternally derived X chromosomes, but also by two sets of maternally derived autosomes (Mann and Lovell-Badge, 1988). It is proposed that the functional differences between parental genomes is determined by gene modification or 'imprinting' presumably imparted during gameto-
genesis (Cattanach and Kirk, 1985; Reik et al. 1987). However, the molecular mechanism by which both parental genomes interact with each other and with the embryonic cytoplasm, are still unknown.

While the genetic aspect of this phenomenon is well documented, the contribution of cytoplasmic events triggered specifically by the sperm has been less studied. It was shown that some cytoplasmic signal present for only a short period of time following artificial activation was able to alter the function of embryonic or somatic nuclei (Czolowska et al. 1984; Szollosi et al. 1986; Szollosi and Szollosi, 1988).

Thus, it was of interest to determine whether any experimentally sustained alteration of the cytoplasmic activity during the earliest stages of oocyte activation was able to influence subsequent developmental events. One of the approaches consists of stimulating the pattern of calcium ion oscillatory activity observed during and after fertilization and initiated by the sperm.

In the present study, two preliminary aspects of this approach were considered. First of all, from a methodological point of view, it was examined whether it was possible to reproduce the Ca\textsuperscript{2+} ionic transients during the parthenogenetic first cell cycle and second, whether the experimental modulation of this cytoplasmic oscillatory mechanism in the absence of male gamete had any affect on the development of the parthenogenetic embryos.

(1) Methodological aspects

The present data demonstrate that the sustained induction of calcium stimuli by electroporation is not only an efficient method of parthenogenetic activation, but offers also a new approach to studying the ionic events responsible for egg activation and their contribution to embryonic development. The response of mammalian oocyte submitted to EF pulses, especially the physico-chemical effects on the plasma membrane are unknown. Nevertheless, control experiments (group A) revealed that oocytes or fertilized eggs were very resistant to high-voltage electric pulse treatment if the electrical current during pulses does not exceed 1 mA, i.e with a very weak concentration of ions in the surrounding medium. It appeared that the egg was insensitive to the electrical signal, but responded to the chemical signal generated by the electrical pulse. Control of the chemical environment during EF in that chamber enabled us for the first time to distinguish between the electrical and the chemical effect of such short-lived signal on the activation of eggs and point out the consequence of the chemical environment under the zona during the pulse.

The evidence that it was indeed the ionic flux and not the EF pulses per se that triggers activation was derived from the experiments in which the rate of activation was directly correlated with the presence of Ca\textsuperscript{2+} in the pulsating medium (group B and C). This study confirms that Ca\textsuperscript{2+} are intimately related to the EF effect (Rodan et al. 1978). Other ions like Mg\textsuperscript{2+} or Na\textsuperscript{+} are far less efficient, but they are also able to activate the rabbit oocyte (unpublished observation).

The response of rabbit oocytes or fertilized eggs to pulsatile electrical stimulation suggests that the micro-breaks induced by EF pulse in the lipid bilayer are of temporary character, similar to those observed in artificial lipid bilayers (Benz and Zimmerman, 1981). Microporation can be repeated many times without adverse effects. The restoration of the original membrane integrity after pulses must occur before the reintroduction of the ion-containing culture medium (which occurred in the stimulating chamber within a few seconds) because the ions reintroduced with the culture medium are unable to generate the chemical signal responsible for activation (control group A).

The putative inward ionic current during the pulse when 10\textmu M of Ca\textsuperscript{2+} was present in the pulsating medium was not measured. But the interaction between activation and pulse duration shown in group B clearly shows that the amplitude of the calcium stimuli is correlated with the duration of the pulse. Although the accuracy of such an indirect control of the calcium signal through the pulse duration has not yet been established, this feature provides a potentially powerful means of generating a variety of chemical signals inside the cytosol with a modulation of amplitude and frequency.

The possibility that the calcium stimulus provides the signal for releasing calcium from the intracellular stores is not demonstrated. However, treatment of oocyte in presence of lithium in the culture medium inhibits the activation of oocytes. Lithium is known to inhibit the final dephosphorylation step of IP\textsubscript{3}, thus reducing the supply of new IP\textsubscript{3} (Berridge, 1989). This result (Ozil, Pesty and Kubiak in preparation) shows that the Ca\textsuperscript{2+} influx via the EF pulse does not directly activate the oocytes but triggers directly or indirectly a process in which IP\textsubscript{3} is involved and leads to release of the Ca\textsuperscript{2+} from internal stores. Such transient oscillations of free Ca\textsuperscript{2+} after EF treatment occur since they are known to regulate the cytokinetic events (Ratan and Shelanski, 1986; Silver, 1989) and generally to be responsible for activation of the egg. The proposed method based on the electroporation technique offers the possibility of studying the dynamics of second messenger activity and its temporal influence in the process of activation (Miyazaki, 1988).

(2) Developmental aspect

The uniformity of the parthenogenetic response of rabbit oocytes submitted to repeated EF stimuli enables us to define the parthenogenetic treatment more like an algorithm of calcium signal than a discrete signal. Recently, it was shown by Stice and Robl (1988) that one EF pulse of 1.6 kV cm\textsuperscript{-1} and 60 \textmu s duration can activate 52\% of aged rabbit oocytes (20–24 h). Only 16\% of them are able to form morulae and blastocysts. In that experiment, the ionic concentration during the pulse was not described. The relatively low efficiency of parthenogenetic activation and development after such a treatment suggests that the chemical signal generated by this EF pulse was rather weak. Nevertheless, a high rate of activation could be obtained with a single EF
pulse of 1.8 kV cm⁻¹ and 2 ms in the presence of 10 μM Ca²⁺ (unpublished results), but the type of parthenogenetic embryo and subsequent development were variable as in other classical methods of activation (unpublished observations).

The present study demonstrates that the temporal regulation of calcium stimuli plays a major role in the process of oocyte activation. The results support the suggestion of Cuthbertson and co-workers (1981) that a biochemical oscillator is present in mammalian oocytes and controls the timing and amplitude of intracellular Ca²⁺ after fertilization.

The results obtained in groups B and C show that for activation a negative exponential reduction of pulse duration is superior to treatment with constant pulse duration. The gradual reduction of pulse duration makes possible a sustained activation without generating side effects on the meiotic events. The mathematical relationship chosen provides a simple way (with three parameters a,b,c) to find the optimal distribution of the duration of 22 pulses.

The most significant fact that emerges from this study is that the level of stimulation affects in vitro developmental potency. The results obtained in group D (treatments I and III) show for the first time that some developmental events like compaction or blastocyst formation can be influenced by the process of activation regulated by Ca²⁺ 2 or 4 days earlier. The culture conditions were the same for both groups. The difference between treatments is related to the total pulse duration and in turn by the strength of the calcium stimulation. This difference may influence the rate and/or the level of the biochemical activity of some cytoplasmic proteins which subsequently regulate the genome expression. Further studies are needed to clarify this fundamental aspect. It is already known that applications of EF pulses to resting somatic cells stimulate the synthesis of DNA (Rodan et al. 1978; Basset and Hermann, 1968; Ozawa et al. 1989) and improved the development of tetraploid rabbit eggs (Ozil and Modlinski, 1986).

The postimplantation viability of parthenogenetic eggs was tested with treatment III. The results demonstrate that parthenogenetic rabbit eggs die at a similar stage of development to parthenogenetic mouse embryos i.e on day 11 of pregnancy. But, in the present series high rates of implantation and embryonic development were obtained till day 10.5 (66.6 %) after appropriate pulsatile EF treatment of the oocytes. The parthenogenetic embryos are smaller than the control, but the development of fetal envelopes, which is known at least in mouse embryos to be controlled by the paternal genome, was proportional to the size of the fetuses. It seems that, in the rabbit with such treatments of freshly ovulated oocytes, the mortality of parthenogenetic embryos is not directly correlated with an abnormal development of the fetal envelopes. The general development of the embryos, the fetus itself, seem slower than the fertilized embryos although the external morphology is comparable. A small fetus with 30 somites was recorded with a similar external morphology to the control fetuses transferred the same day in the opposite uterine horn (Fig. 6). Nevertheless, for the same treatment and the same type of genome (parthenogenetic diploid), some embryos are alive and others are abnormal with a variety of morphological abnormalities. Those abnormalities appear step by step from day 8 and reach all the fetuses on day 12 of pregnancy. It is difficult to determine which genetic and/or epigenetical factors were responsible for this phenomenon and when they took place, but the routine production of parthenogenetic fetuses will enable us by biochemical studies to understand why parthenogenetic fetuses died before the first third of pregnancy.

In conclusion, the process of activation is not a time-limited event in the development, but rather a process that is sustained by a permanent interaction between sperm and oocyte. Ca²⁺ plays a central role in this process. The present method will be useful for investigating the developmental effects caused by the intracellular Ca²⁺ oscillations during and after oocyte activation.

This process of activation will also be a tool well-fitted to dissociate the study of the major two functions intimately conjugated during the process of fertilization i.e to re-establish the diploid status of the egg by introduction of a paternal haploid genome and to trigger the embryonic development. Introduction of a diploid genome into an egg is under the control of the nuclear transfer technique, but the development of such hybrids is generally poor. The question arises whether the introduced nucleus is able to promote normal development and also to reproduce the process of activation trigger by the sperm that appears crucial for the development?

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