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Artificial Activation of Pig Oocytes Arrested at Meiotic Metaphase II

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제2감수분열 중기에서 발달정지된 돼지 난자의 인위적 난활성

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요 약

포유동물의 난자는 제 2 감수분열 중기에서 배란되어 수정된다. 일반적으로 포유동물에서의 수정은 inositol triphosphate (IP_3) 또는 cyclic adenosine diphosphoribose (cADPr)에 의해 조절되어지는 세포질내 칼슘변화에 의해 일어난다. 난활성이 일어나면 세포질내 고농도의 MPF (maturation promotion factor)는 감소하고, 전핵이 형성되고, cytoskeleton이 재구성되고, 단백질합성이 조절되어진다. 이러한 모든 과정이 정상적으로 일어난 후 수정란은 종특이한 발달경로로 배발달을 시작한다. 본 논문에서는 돼지난자의 인위적인 난활성 유도방법에 대해서 설명하고자 한다. 신호전달(signal transduction) 경로에 의한 하나의 기전이 돼지난자에서 확인되었는데, 이 경로의 자극은 난활성으로 이루어진다. 또한 전기자극에 의한 난활성방법에 의해 12일까지 정상배 발달이 이루어진다고 보고되고 있다. 향후 연구에서는 정자가 어떠한 기전에 의해서 배발달을 시작하게 하는지를 밝혀내야 한다고 본다.

I. INTRODUCTION

By day 30 of pig embryogenesis there is a 30% to 40% loss of conceptuses (Wilmut et al., 1986). It is not clear exactly when these losses occur. Relatively little is known about fertilization and early development in pigs. A better understanding of the earliest events after ovulation may have a significant impact on developing technology for *in vitro* fertilization, *in vitro* development, transgenics, cloning by nuclear transfer and non-surgical embryo transfer. This lab has been very interested in *in vitro* fertilization (Kim et al., 1996c; Funahashi et al., 1996), embryo development (Schoenbeck et al., 1992; Prather, 1993), cloning (Prather, 1996) and more recently has developed an instrument for non-surgical embryo transfer (Li et al., 1996a). Since all of these technologies deal with the unfertilized oocyte or the early embryo, it is imperative that fertilization (or mimicking fertilization as for nuclear transfer) occur normally. Unfortunately, very little is known about the events that occur at fertilization in the pig. We do know that in response to fertilization cortical granules are released, repetitive calcium transients occur (Sun et al., 1992), H1 kinase activity and glutathione levels decrease (Funahashi et al., 1994), and a 25 kDa protein is dephosphorylated to 22 kDa (Ding et al., 1992). Many of these events can be induced with an

electric pulse (Prather et al., 1991; Ding et al., 1992) and development to the filamentous blastocyst stage can result (Jolliff and Prather, 1997).

In this review we hope to illuminate many of the cellular changes that occur at fertilization or activation, and describe the mechanisms that may regulate activation. In addition, the degree of development that may be expected will be described and finally the review will end with how this information may be applied and where to look for the next benefits.

Ⅱ. INTRACELLULAR CHANGES AT FERTILIZATION

In general, mammalian oocytes are ovulated arrested at metaphase Ⅱ of meiosis. This arrest is maintained by maturation promoting factor (MPF) and cytostatic factor (CSF). MPF is composed of $p34^{cdc2}$ and cyclin, while the active component of CSF is the product of the proto-oncogene c-mos. In the normal process of fertilization, the oocyte is released from the metaphase Ⅱ arrest and proceeds to the first interphase. Temporally correlated with this progression to interphase a number of early events occur: 1) sperm-oocyte fusion, 2) increase in intracellular calcium concentration ($[Ca^{2+}]_i$), 3) degradation of cyclins, and of Mos, 4) cortical granule release, 5) zonae pellucidae hardening, and 6) repetitive oscillations of $[Ca^{2+}]_i$. There are two major theories regarding what causes the initial increase in $[Ca^{2+}]_i$, which is one of the first measurable cellular response to fertilization.

1) The sperm binds a plasma membrane associated receptor that is coupled to either a G protein- or a tyrosine kinase-coupled mechanism of signal transduction (Kline et al., 1988; Williams et al., 1992; Berridge, 1993; Yim et al., 1994; Shilling et al., 1994; Moore et al., 1993; Lindsay and Clark, 1994). Data in guinea pig also suggest that ligand-receptor-effector interactions (integrins-disintegrins) might mediate sperm-egg binding, fusion, and egg activation (Blobel et al., 1992; Almedia et al., 1995). While stimulation of these pathways is capable of inducing most of the early and late events associated with fertilization, it does not mean that these pathways are used during fertilization.
2) The sperm cell carries in a compound that stays associated with the chromatin. This compound, an oscillogen, causes the initial burst of intracellular Ca^{2+} as well as the repetitive oscillations (Dale, 1988; Stice and Robl, 1990; Swann, 1994; Homa and Swann, 1994) and this factor may stay associated with the chromatin for a few cleavage divisions (Kono et al., 1995; Zernicka-Goetz et al., 1995).

A third possibility is that both of the above mechanisms are involved in the pathway of sperm induced oocyte activation. As a review of signal transduction systems, we have included Fig. 1. In this model, a ligand binds a receptor on the plasma membrane, either a G protein- or tyrosine kinase-coupled receptor. This transduces a signal to either phospholipase C-(for tyrosine kinase or phospholipase C-J for the G protein. Either will cleave phosphatidylinositol biphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 then acts upon IP_3 receptors in the endoplasmic reticulum and causes an increase in $[Ca^{2+}]_i$. The DAG acts on protein kinase C (PKC). This proposed scheme would result in a single burst of intracellular calcium. In sea urchins, it is thought that cADPr is also involved in the release of $[Ca^{2+}]_i$, but it is not clear how this is achieved.

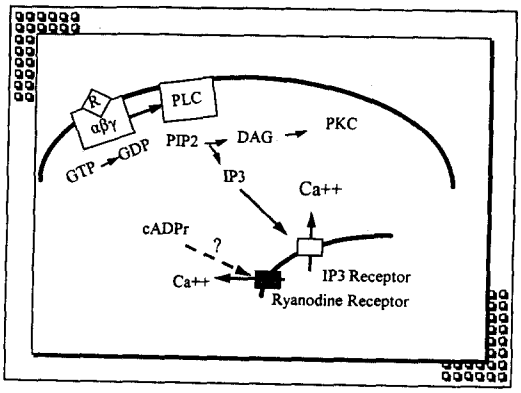

Fig. 1. Calcium release mechanisms proposed for pig oocytes. In this model, a ligand binds a receptor(R) on the plasma membrane. This transduces a signal via the G protein to phospholipase C-β which will cleave phosphatidylinositol bisphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol(DAG). The IP_3 then acts upon IP_3 receptors in the endoplasmic reticulum and causes an increase in $[Ca^{2+}]_i$. The DAG acts on protein kinase C (PKC). This proposed scheme would result in a single burst of intracellular calcium. In sea urchins, it is thought that cADPr is also involved in the release of $[Ca^{2+}]_i$, but it is not clear how this is achieved.

Calcium Responses.

Fertilization induces a series of events in the oocyte, which is generally referred to as "activation" (Kopf et al., 1989). The response to fertilization in some species is a single wave of $[Ca^{2+}]_i$, while in mammals a series of oscillations is initiated. Interestingly, cells may be able to respond differently to repetitive than to monotonic $[Ca^{2+}]_i$ increases and the frequency may also influence the response (Meyer and Stryer, 1991). A large $[Ca^{2+}]_i$ release followed by a series of periodic $[Ca^{2+}]_i$ releases occurs in mammalian and ascidian eggs (McDougall and Sardet, 1995). Interestingly, while the Ca^{2+} responses after fertilization are somewhat similar, the ascidian oocyte prior to fertilization is arrested at meiotic metaphase I while mammals tend to arrest at metaphase II. In human oocytes, sperm-oocyte fusion results in the intracellular release of Ca^{2+} from a point on the periphery. This Ca^{2+} increase then travels around the periphery and finally reaches the center of the cytoplasm. The peak $[Ca^{2+}]_i$ is always higher in the center of the oocyte than at the periphery (Tesarik et al., 1995).

As follow-up on the above suggestion that the type of Ca^{2+} stimulation is important, it has been shown in mouse oocytes that meiotic resumption of parthenogenetically activated oocytes is dependent on the level of $[Ca^{2+}]_i$ (Vincent et al., 1992). In fact, mimicking the normal pattern of $[Ca^{2+}]_i$ oscillations resulted in an increase in the development of rabbit oocytes (Ozil, 1990) and the rate of pronuclear formation in ascidian and mouse oocytes (McDougall and Sardet, 1995; Ozil and Swann, 1995). Thus the type of Ca^{2+} stimulation is very important for promoting subsequent development.

Electrophysiological studies showed that the first detectable change in the sea urchin egg after sperm-egg contact is an inward current (the sperm-current). It has an abrupt onset and depolarizes the egg membrane potential causing an action potential (Jaffe, 1976; Dale et al., 1978; Chambers and de Armendi, 1979). Depolarization induces calcium entry through voltage-gated channels of the plasma membrane and thus causing a rise in $[Ca^{2+}]_i$ below the membrane (Whitaker and Swann, 1993). This calcium influx also sets off a small calcium induced calcium release (CICR). The large fertilization calcium explosion proper starts some

15 seconds later at the point of sperm attachment and crosses the egg in about 20~30 sec (Eisen et al., 1984; Swann and Whitaker, 1986). This $[Ca^{2+}]_i$ release is the direct cause of the wave of cortical granule exocytosis and resultant fertilization envelope elevation that also travels across the egg at a velocity of 5~10 μ m/second (Jaffe, 1983; McCulloh and Chambers, 1991). This progressive exocytosis, or envelope elevation, is referred to as the fertilization wave.

After the sperm-induced current a second inward current can be measured in the fertilized eggs. It is called the activation current and it coincides with the fertilization wave and calcium transient found at fertilization (David et al., 1988). The interval between the start of the two currents varies from 7 to 30 seconds (Shen and Steinhardt, 1984; Swann and Whitaker, 1990).

The fertilization calcium waves in frog and sea urchin eggs involve the modulated release and re-uptake of calcium from intracellular stores, i. e. the endoplasmic reticulum (Terasaki and Sardet, 1991). In somatic cells the release of calcium occurs through a family of related calcium channels that are gated by the phosphoinositide messenger, IP3 (Ross et al., 1989; Furuichi et al., 1989; Mignery et al., 1989); by calcium itself (Lai et al., 1988; Finch et al., 1991); and possibly by a newly discovered and potent calcium-mobilizing agent cADPr (Galione et al., 1991).

After the initial calcium release from intracellular stores spatial waves and temporal oscillations form due to the explosive positive feedback mechanisms built into the calcium signalling system (Kacser, 1955). The positive feedback can operate as the result of calcium-stimulated production of IP3 (Whitaker and Irvine, 1984; Meyer and Stryer, 1988), through stimulation of ryanodine-sensitive calcium release channels by calcium itself (CICR; Endo, 1977; Lai et al., 1988; Takeshima et al., 1989) or through IP3-induced alterations in the sensitivity of IP3-gated release channels to calcium (Finch et al., 1991; Bezprovzanny et al., 1991).

Besides the increase in the intracellular free calcium there is another ionic change in the sea urchin cytoplasm that plays a role in triggering development. It is the sustained rise in intracellular pH (Johnson and Epel, 1976; Shen and Steinhardt, 1978); in Lytechinus pictus pH rises from 6.8 to 7.3 over the first five minutes at fertilization. This pH increase which is caused by the activation of a plasma membrane Na^+/H^+ antiporter (Johnson and Epel, 1976; Payan et al., 1983) is important in acting co-operatively with the calcium transient to stimulate protein and DNA synthesis (Whitaker and Steinhardt, 1982). Since calcium ionophores cause a rise in the intracellular pH similar to that seen at fertilization (Whitaker and Steinhardt, 1981) it appears that the Ca^{2+} transient leads to the pH increase. These two ionic changes finally cause the biochemical and morphological changes in the oocyte that are termed the early and late events of fertilization. Such changes in pH have not been observed in mammals (Kline and Zagray, 1995; Ben-Yosef et al., 1996; Phillips and Baltz, 1996).

An increased turnover of poly-phosphoinositides occur within the first 5 seconds of fertilization in sea urchin eggs (Turner et al., 1984). The hydrolysis of PIP_2 generates the second messenger IP_3 which releases Ca^{2+} from intracellular stores (Berridge, 1987). Since microinjection of IP_3 in sea urchin eggs causes an exocytosis, envelope elevation, pH_i rise (Whitaker and Irvine, 1984) and also an increase in the $[Ca^{2+}]_i$, it is suggested that IP_3 is the cell messenger responsible for the sperm-induced $[Ca^{2+}]_i$

transient. However, at fertilization there is a considerable delay between the start of the sperm-induced current and the calcium wave (called the latent period) which is thought to represent the time required for the spermatozoa to cause enough IP_3 production or enough local $Ca^{2+}{}_i$ increase to trigger the wave (Swann and Whitaker, 1990). It is not totally clear how the sperm causes these initial changes.

1. Meiotic resumption without calcium

There have been a number of reports of meiotic resumption without $[Ca^{2+}]_i$ release. In the mouse, inhibition of protein synthesis results in the resumption of meiosis without the $[Ca^{2+}]_i$ increases observed normally at fertilization (Moses and Kline, 1995a). Inhibition of protein kinases in both the mouse and pig also results the resumption of meiosis (Mayes et al., 1995; Rickords et al., 1992), but without cortical granule release in pigs (Mayes et al., 1995) and without a change in $[Ca^{2+}]_i$ in mice (Rickords et al., 1995). Similarly, treatment of Xenopus oocytes with 6-dimethyl aminopurine (6-DMAP) results in meiotic resumption without a $[Ca^{2+}]_i$ transient (Zhang and Masui, 1992), as does 6-DMAP treatment of molluscan oocytes (Neant and Gurrier, 1988). In contrast to what might be expected, Moses and Kline (1995b) showed that phorbol 12-myristate 13-acetate (PMA) induced oocyte activation can be independent of $[Ca^{2+}]_i$. This suggests that some protein synthetic and phosphorylation/dephosphorylation events are occurring at fertilization after the $[Ca^{2+}]_i$ transient(s).

While the inclusion of protein kinase inhibitors (PKIs) in a scheme to activate oocytes may on the surface appear counter-productive, PKI has been shown to play a role in parthenogenetic activation in many systems. For example, Swann et al., (1989) showed in hamster oocytes that GTP-K-S (a non-hydrolyzable form of GTP) induced hyperpolarizations were inhibited by PKC agonists 12-O-tetradecanoyl phorbol acetate (TPA) and 1,2-dioctanoyl-sn-glycerol (DiC8), while being enhanced with sphingosine. Sphingosine competes with diacylglycerol phorbol dibutyrate and Ca^{2+} and inhibits PKC activation by other lipids (Hannun et al., 1986; Oishi et al., 1988). Interestingly, neither TPA nor sphingosine altered the IP3-induced hyperpolarizations (Swann et al., 1989); thus the PKIs may be acting upstream from the IP_3 response. An endogenous PKI (cAMP-dependent) is present in the nucleus of tissue culture cells. Its quantity and location is cell cycle dependent and becomes localized in the nucleus at the G2/M boundary. Inhibition of its function with antibodies prevents cell cycle progression (Wen et al., 1995). Together, the above data suggests that PKI may be a normal part of cellular function at the G2/M/G1 transition.

2. Maturation promoting factor and cytostatic factor

A report by Watanabe et al., (1991) described the activity of MPF and the c-mos gene product Mos during activation. MPF degradation after activation was measured by evaluating cyclin B1 and B2. Cyclin B1 began degradation between 5 and 10 min after activation. Cyclin B2 began degradation between 10 and 15 min after activation; whereas Mos, also known as cytostatic factor (CSF: Sagata et al., 1989), underwent a size shift (dephosphorylation) at 15 min and almost complete degradation after 20 min post-activation. In addition, MPF activity disappeared before the disappearance of Mos induced activity. Thus MPF inactivation is probably independent of CSF inactivation. Cytoplasmic free Ca^{2+} seems to be required for the inactivation of MPF. One model for the release of

meiosis suggests that the fertilization induced Ca^{2+} release sequentially inactivates MPF and CSF (Watanabe et al., 1991; Lorca et al., 1993). Inhibitors of the calmodulin-dependent protein kinase Ⅱ prevent cyclin degradation even after Ca^{2+} elevation (Lorca et al., 1993). In addition, the calmodulin-dependent protein kinase Ⅱ can release the cyclin degradation machinery in the absence of Ca^{2+}. This cyclin degradation machinery appears bound to a myosin light chain kinase. Mos, on-the-other-hand, is degraded through the calpain-dependent pathway (Watanabe et al., 1989).

The product of the c-mos proto-oncogene functions not only as an initiator of oocyte maturation but also as a component of cytostatic factor that causes the natural arrest of the unfertilized egg at the second meiotic metaphase. Mos is a cytoplasmic serine/threonine kinase. Mos activity is thought to be necessary for the meiotic arrest (Hashimoto et al., 1994; Colledge et al., 1994), by preventing the ubiquitin-dependent degradation of mitotic cyclins and thus inactivation of MPF (Murray et al., 1989; Glotzer et al., 1991). Other roles of c-mos may be to maintain high MAP kinase activity in meiosis (Nebreda and Hunt, 1993), and to prevent undesirable DNA replication or parthenogenetic activation before fertilization (Furuno et al., 1994).

Since there are species specific differences in the response to fertilization, i. e. Ca^{2+} response, and since different species arrest at different stages of meiosis, a brief overview of some of the specifics learned from the mouse will be presented prior to the pig data.

Ⅲ. ACTIVATION OF MOUSE OOCYTES

The development of the ability to respond to sperm, IP_3, ionomycin, or ryanodine induced activation by producing sustained $[Ca^{2+}]_i$ oscillations occurs during late oocyte maturation (Carroll et al., 1994; Mehlmann and Kline, 1994; Jones et al., 1995). This competence to produce sustained Ca^{2+} oscillations is also correlated with the ability to undergo cortical granule exocytosis (Ducibella and Buetow, 1994). Monoclonal antibodies to the IP_3 receptor block the inositol-induced calcium release (IICR), and this shows that IICR is essential in the initiation, propagation and oscillation of the sperm-induced Ca^{2+} rises (Miyazaki et al., 1993). Injection of antibodies to the IP_3 receptor also obstructs the block to poly-spermy, inhibits the fertilization-induced drop in H1 kinase activity and pronucleus formation as well as the later events associated with fertilization (Xu et al., 1994).

Interestingly, the ability of ryanodine injected into oocytes to increase $[Ca^{2+}]_i$ is strain-specific (Swann, 1992; Jones et al., 1995), and thus may explain the results of Kline and Kline (1994) who found no evidence for a ryanodine-sensitive store of Ca^{2+} in mouse oocytes. A similar dichotomy may occur in cattle (Yue et al., 1995; Fissore et al., 1995).

When mouse oocytes are treated with TPA, sustained oscillations in $[Ca^{2+}]_i$ are observed about 20 min after treatment (Cuthbertson and Cobbold, 1985). These $[Ca^{2+}]_i$ oscillations last about 4 h, similar to those observed in normally fertilized oocytes. It was proposed that TPA mimics DAG and causes the $[Ca^{2+}]_i$ to be released via a PKC pathway. PMA does not significantly affect the egg membrane permeability to Ca^{2+} (Colonna et al., 1989), thus $[Ca^{2+}]_i$ changes are due to release from intracellular stores. Phorbol esters can indirectly induce zona pellucida protein 2 (ZP2) and ZP3 to be modified to become similar to ZP2 ($ZP2_f$) and ZP3 ($ZP3_f$) isolated from zygotes (Endo et al., 1987), but only after 40 min of exposure (Colonna et al.,

1989). In addition to the $[Ca^{2+}]_i$ transients, there is also a release from the metaphase II arrest; however, the second meiotic division is suppressed in a dose dependent fashion. When oocytes are exposed to PMA for as little as 2 h prior to fertilization, the second polar body is not released (Niemierko and Komar, 1985). While all the above evidence suggests that phorbol esters activate oocytes by a Ca^{2+} mechanism, Moses and Kline (1995b) present evidence to suggest that phorbol ester induced activation is independent of $[Ca^{2+}]_i$. Thus activation by PKC provides only part of the story.

Ⅳ. OTHER METHODS OF ARTIFICIAL ACTIVATION

Release from the block at metaphase II can be induced by a wide variety of stimuli. These include electric shock in Ca^{2+} containing medium, IP_3 injection, heat treatment, ethanol treatment, inhibition of protein synthesis, treatment with phorbol esters, treatment with protein kinase inhibitors (PKIs), etc. Many of these stimuli are species specific; for example: protein synthesis inhibition alone does not result in meiotic resumption in amphibians (Ziegler and Masui, 1976; Zhang and Masui, 1995) or rats (Zernicka-Goetz et al., 1993) as in mice (Siracusa et al., 1978; Clarke and Masui, 1983) and humans (Balakier and Casper, 1993). Recently attention has focused on combination treatments such as ionophore treatment followed by PK inhibition, or ionophore treatment followed by inhibition of protein synthesis, or electrical pulse followed by DiC_8 (a membrane permeable diacylglycerol; Schoenbeck et al., 1993). Such combinations are designed to attack two different pathways, 1) provide the Ca^{2+} transient, and 2) follow up with blocking cyclin production or inhibiting phosphorylation. Sequential treatment of mouse oocytes with ionophore followed by protein synthesis inhibition results in uniformly haploid parthenogenones (Hagemann et al., 1995). Similar treatments in cattle and pig oocytes have a synergistic effect on the percent that form pronuclei (Presicce and Yang 1994a, b; Nussbaum and Prather, 1995).

Ⅴ. ACTIVATION OF PIG OOCYTES

In general, the pig oocyte appears to be similar to the oocytes of many other mammals. The main findings will be summarized below. There are equal amounts of cyclin B2 and $p34^{cdc2}$ in growing oocytes (Christmann et al., 1994), but cyclin B1 is not synthesized until germinal vesicle breakdown (Naito et al., 1995). Differential synthesis may relate to function as B1 localizes to microtubules and B2 localizes to the Golgi apparatus (Jackman et al., 1995). c-Mos has been identified in the pig (Newman and Dai, 1996), but there has been little characterization during fertilization.

Microinjection of GTP-γ-S, IP_3 or $CaCl_2$ induces many of the early and late events associated with fertilization-induced activation (pronuclear formation, cortical granule release, dephosphorylation of the 25 kDa protein(s), decrease in histone H1 activity, development to blastocyst) while control injections of GDP-β-S, IP_2 or $MgCl_2$ do not induce activation (Machàty et al., 1995, 1996, 1997a). The development of the ability to respond to injection of IP_3 or cADPr occurs during maturation of the oocyte, with maximal responses obtained at meiotic metaphase II (Machàty et al., 1997a). While these results suggest that the signaling pathway is present and capable of activation, the final test of the pathway would be by the injection of the message encoding a G protein-coupled receptor followed

by stimulation of that receptor. When a message encoding the rat M1 muscarinic acetylcholine receptor was introduced into the oocyte, followed by translation and acetylcholine treatment, the oocytes exhibited a $[Ca^{2+}]_i$ transient, a drop in histone H1 kinase activity, they formed pronuclei, dephosphorylated the 25 kDa protein and continued to the blastocyst stage (Machàty et al., 1997b; Kim et al., unpublished)

Electrical poration of Ca^{2+} has been the standard by which other methods have been measured (Prather et al., 1989, 1991). An electric pulse in the presence of Ca^{2+} results in cortical granule release and the dephosphorylation of the 25 kDa protein (Sun et al., 1992; Mayes et al., 1995). While there is no evidence that a pH change occurs at fertilization in the pig, it is clear that electrically-induced activation is pH dependent (Prather et al., 1991). Glutathione levels decrease after fertilization, but do not decrease after electrical stimulation (Funahashi et al., 1995) or after activation by GTP-γ-S injection (Funahashi et al., 1996). This sperm-induced decrease may be due to the introduction of γ-glutamyl transpeptidase by the sperm (Funahashi et al., 1996). An electric pulse results in a single Ca^{2+} transient which is the result of an influx of extracellular Ca^{2+}. Electroporation in zero Ca^{2+} medium results in no Ca^{2+} flux and no resumption of meiosis (Sun et al., 1992). This may be in contrast to the mouse where activation was not dependent upon extracellular Ca^{2+} (Tombs et al., 1992). While the authors (Tombs et al., 1992) have observed a 4-fold increase in the $[Ca^{2+}]_i$ of the mouse oocytes during maturation and use that as an explanation for the Ca^{2+} independence, we have found that removal of extracellular Ca^{2+} (and other ions?) requires extensive rinsing in purified water. Even a small amount of residual Ca^{2+} can result in activation of pig oocytes in supposed Ca^{2+}-free conditions (unpublished observations). Electrostimulation results in a wave of Ca^{2+} release starting at the positive electrode and spreading over the entire oocyte at 5~10 μm/sec. This release of $[Ca^{2+}]_i$ is dependent upon extracellular Ca^{2+}, DC field strength and DC pulse duration (Sun et al., 1992). Sperm induced $[Ca^{2+}]_i$ spikes continue for many h and tend to increase in length as time post-fertilization increases (Sun et al., 1992). Sequential treatment of an electric pulse and protein synthesis inhibition results in enhanced percentages of pronuclei and subsequent development to blastocyst (Nussbaum and Prather, 1995). Protein synthesis inhibition alone is not sufficient to induce parthenogenesis as in amphibians (Ziegler and Masui, 1976; Zhang and Masui, 1995) or rats (Zernicka-Goetz et al., 1993).

The use of aged oocytes has been one method of increasing the percentage of eggs that will activate; however, aging results in the chromatin moving out of the cytoplasmic region where microfilaments are thick and is correlated with the formation of numerous micronuclei often observed in parthenogenetically activated oocytes (Kim et al., 1996b, 1996d).

While the above description is similar to most mammals, a few papers suggest that protein kinase inhibition induces activation in pig oocytes. High (Mayes et al., 1995) and low (Prather et al., 1997) concentrations of H7 for a short period of time cause pronuclear formation and the characteristic dephosphorylation of the 25 kDa protein, while iso-H7 has little effect (Mayes et al., 1995). Similarly, staurosporine (Rickords et al., 1992) induces pronuclear formation, however there are no $[Ca^{2+}]_i$ changes (Rickords et al., 1995). Cortical granule release was not evaluated, but may be similar to H7 treatment which does not cause cortical granule release. This is

in contrast to what occurs in Xenopus, i. e. treatment with the sphingolipids (staurosporine, sphingosine) causes both a Ca^{2+} transient and progression to first interphase.

Development.

Developmental potential after activation of the oocyte is one of the important endpoints. Many of the studies that show pronuclear formation after activation either can not show further development or have not attempted further development. It is clear that just because high percentages of pronuclear formation can be attained, subsequent development does not always follow. An excellent example of this is one of our papers (Mayes et al., 1995). It is shown that very high percentages of single pronuclear formation can result after treatment with H7, a protein kinase inhibitor, but these oocytes are not capable of developing to the blastocyst stage. Until recently all of the studies that showed good development to blastocyst were conducted after transfer to a gilt. Thus it was not clear if the inability of development to continue *in vivo* was due to the *in vitro* maturation conditions for the oocyte, or due to the culture conditions after activation. Since the activated oocytes that are placed *in vivo* do develop, our culture conditions after activation are likely limiting; however, recently we have changed the *in vitro* maturation conditions (Li and Prather, unpublished) and can obtain development to blastocyst using the same post-activation conditions that previously did not result in development to blastocyst. Thus it appears that both the *in vitro* maturation conditions and the *in vitro* conditions for bleavage and blastocyst formation were limiting.

An additional reason that these parthenogenetic eggs do not develop may be due to genomic imprinting. Genomic imprinting has been defined as the silencing of a gene on one of the parental chromosomes. For example, if a gene is maternally imprinted, then if the gene is processed through maternal gametogenesis, then the gene will be nonfunctional in the offspring (de Groot and Hochberg, 1993). Some specific examples in mammals include the insulin like growth factors. Our initial studies suggests that imprinting may also play a role in early pig parthenogenotes (Li et al., 1996b).

Finally, the desired endpoint of artificial activation is the ability to direct term development. As we have seen above, this is unlikely since specific gene products will be missing. However, it is of value to know how far such embryos will develop. In mice parthenogenetic embryos will develop to midimplantation before they die. They have a relatively well developed fetus, with a more poorly defined placenta (Surani et al., 1984). Similar development to day 35 has been reported in cattle (Fukui et al., 1992). Recently we have shown that parthenogenetically activated pig oocytes can, by day 14, elongate and have an embryonic disc (Jolliff and Prather, 1997).

While these embryos can develop, it is not clear how the mitotic apparatus is organized. In mice the microtubule organizing centers come from the egg and are not inherited from the sperm, while in the pig and cow the sperm brings the centrosome into the unfertilized egg and this centrosome organizes the microtubules for the first mitotic division (Schatten, 1994). While, in pigs, a centrosome is inherited from the sperm and it organizes the microtubules at fertilization, cytoplasmic microtubule organizing centers can be induced with taxol (Kim et al., 1996a). Further research is needed to identify the microtubule organizing activity in these embryos.

Ⅵ. APPLICATIONS

Adequate methods of artificial activation would be very useful for the production of clones by nuclear transfer. This method of cloning involves taking a membrane enclosed nucleus and fusing it to an enculated oocyte. Two things must occur after fusion: 1) the nucleus must be reprogrammed to recapitulate development, and 2) the oocyte must be stimulated such that it behaves as though it had been fertilized (Prather, 1996). Similarly, by dissecting apart the mechanisms that will lead to the breaking of the meiotic arrest we may gain an insight to the normal process(es) of the sperm uses at fertilization. With this understanding we may be able to modify the current conditions for *in vitro* fertilization.

Ⅶ. SUMMARY

Mammalian eggs are ovulated arrested at meiotic metaphase Ⅱ until fertilization. Generally in mammals, fertilization results in a series of intracellular calcium oscillations that are mediated by inositol triphosphate (IP_3) or cyclic adenosine diphosphoribose (cADPr). The high levels of maturation promotion factor (MPF) within the cell decrease, pronuclei form, the cytoskeleton is reorganized and proteins are post-translationally modified. If all is normal, the newly formed embryo initiates the developmental program specific to that species. Artificial methods of producing these effects in pig oocytes are discussed. One potential mechanism mediated via a signal transduction pathway is present in pig oocytes. Stimulation of this pathway leads to the early events following fertilization, and electrical stimulation leads to apparently normal development to day 12. Further studies are needed to determine which mechanism(s) the sperm uses to initiate development.

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