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Review

Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer

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Introduction

Since the birth of the first mammal, a sheep, by nuclear transfer (Willadsen, 1986) numerous studies have been carried out to elucidate the developmental mechanisms in NT embryos. The potential of this technology was envisioned for the study of developmental processes during embryogenesis, but also for the production of genetically modified animals for commercial purposes. Scientists stimulated by these ideas performed a large number of experiments during the last decade that gave rise to substantial achievements in this field of research. The recipient oocyte (cytoplast), the donor cell (karyoplast) and their interplay were the protagonists of this research. While the perspectives of this technology are very promising, the efficiency of NT is still very low. Several questions related to early events during embryonic development including oocyte activation, chromatin remodelling, gene expression, and cell cycle regulation remain to be answered. Activation of the recipient oocyte after NT is a key step in the cloning procedure. The sperm naturally initiates oocyte activation during fertilisation. However, numerous procedures have been developed to artificially activate oocytes. The purpose of this review is to provide an update of the known events characterising sperm-mediated oocyte activation in mammals, as well as the artificial activation protocols used at present for the generation of parthenogenetic and nuclear transfer embryos.

Activating the MII arrested oocyte

Mammalian oocytes (except of canine oocytes which are arrested at prophase of meiosis I) are arrested at MII after ovulation and complete meiosis after fertilisation. When removed from secondary follicles, immature oocytes undergo spontaneous maturation and remain arrested at MII stage until fertilisation. The MII arrest is characterised by a high MPF activity (Nurse, 1990). MPF, a heterodimer of cdc2 and cyclin B, is stabilised by CSF, which is composed of at least three proteins: Mos (Sagata, 1989), MAPk

Abbreviations used in this paper: BrdU, 5-bromo-2´-deoxyuridine; Ca^{2+}, calcium; cdk, cyclin-dependent kinase; CDKI, cdk inhibitor; CSF, cytostatic factor; G protein, GTP binding protein; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; MAP, mitogen activating protein; MAPk, MAP kinase; MII, metaphase II; MPF, maturation promoting factor; NT, nuclear transfer; PKC, protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PTK, protein tyrosine kinase; RyR, ryanodine-sensitive receptors; SE, sperm extract; SOAF, sperm-borne activation factor; 6-DMAP, 6-dimethylamino purine.

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(Haccard *et al.,* 1993), and p90^{Rsk} (Bhatt and Ferrell, 1999; Gross *et al.*, 1999). The sperm induces the release from the meiotic arrest by a signal transduction pathway not fully understood; however, as we will discuss below, calcium, MPF and its stabilising molecules play a determinant role during oocyte activation. The proper interpretation of the sperm-initiated events and oocyte response(s) during fertilisation will give new clues for the development of activation protocols mimicking the physiological fertilisation process.

Ca^{2+} as the trigger of oocyte activation

The binding of the sperm to the oocyte plasma membrane induces intracellular Ca^{2+} release in the oocyte during fertilisation (Lawrence *et al.*, 1997). The initial rise of free cytoplasmic Ca^{2+} starts from the site of sperm penetration and expands as a wave through the oocyte (Jaffe 1983; Whitaker and Swann, 1993). While one Ca^{2+} transient is registered in echinoderm, fish, and frog oocytes (Jaffe, 1983), repetitive calcium oscillations that last several hours are observed in mammals (Miyazaki *et al.*, 1993; Sun *et al.*, 1994; Nakada and Mizuno, 1998). This has been shown in mice, hamster, rat, rabbit, porcine, bovine, and human oocytes (reviewed by Jones, 1998). The first calcium wave originates from the penetration site (Miyazaki *et al.*, 1986), whereas subsequent oscillations arise in the cortical region of the vegetal hemisphere with a non-wave type uniform calcium rise in mouse oocytes (Deguchi *et al.*, 2000). Calcium oscillations are of low frequency, and appear at intervals of 6-31 minutes in mouse (Deguchi *et al.*, 2000) and 8-25 minutes in bovine oocytes (Fissore *et al.*, 1992). The interval of Ca^{2+} transients is prolonged with time and last for several hours, until pronuclear formation in mouse oocytes (Kline and Kline, 1992; Deguchi *et al.*, 2000). These Ca^{2+} oscillations last for 22 hours in bovine with a decline in the amplitude by 12-15 hours (Nakada *et al.,* 1995). It is still not clear how the Ca^{2+} oscillations are regulated; however, a recent study in ascidians demonstrates the importance of cyclin B for the maintenance of Ca^{2+} oscillations. When cyclin B decreases oscillations terminate, however, when a stabilised form of cyclin B is injected, the calcium oscillations continue indefinitely. The sperm-triggered calcium oscillations are positively regulated by the MPF activity in the ascidian oocyte, independent of the MAPk activity (Lavasseur and McDougall, 2000). These findings confirm early observations of Jones *et al.* (1995) where mouse activated oocytes treated with colcemid (a microtubule inhibiting drug) maintain calcium oscillations in the presence of a metaphase plate, suggesting a cell cycle modulation of sperm-induced Ca^{2+} transients (Jones *et al.*, 1995). In contrast, when colcemid treated mouse oocytes are incubated in the presence of the MPF inhibitor roscovitine, calcium oscillations are suppressed after the two initial calcium spikes (Deng and Shen, 2000). Thus, inhibition of MPF activity in MII oocytes inhibits sperm-induced calcium oscillations, indicating that MPF plays an important role in regulation of the cytoplasmic Ca^{2+} excitability in mouse oocytes (Deng and Shen, 2000).

There are at least three substantial differences between mammals and species that generate a single calcium transient during fertilisation (e.g. sea urchins, frogs and fish). First, in the case of sea urchin, where meiotic maturation is accomplished before fertilisation, a single calcium transient is sufficient to activate the egg (Stricker, 1999). In contrast, mammalian oocytes are arrested at metaphase II and they need to resume meiosis after fertilisation. When a single Ca^{2+} pulse is applied in a mouse oocyte, completion of meiosis is observed, while no pronuclear formation takes place. Instead of a pronucleus, a metaphase plate (or MIII) forms after extrusion of the second polar body (Kubiak *et al.*, 1993). The second difference is the timing of events following meiotic resumption. While a frog oocyte initiates pronuclear formation within minutes after sperm penetration, in mammals this is observed after 2-6 hours depending on the species (mouse: 3-4 hours, Krishna and Generoso, 1977; bovine: 4-6 hours, Liu and Yang, 1999). One single Ca^{2+} transient induces the degradation of MPF and later of CSF in frogs; however, a single Ca^{2+} transient is not sufficient to induce definitive MPF degradation in young matured mouse oocytes (Kubiak *et al.*, 1993). Third, since fertilisation is an external process in frogs, the metaphase II arrested oocyte and the sperm get in contact within a short period of time. In contrast, mammalian fertilisation is internal, that means a variable length of time between ovulation and fertilisation, suggesting that meiotic arrest in mammals is essential to ensure successful fertilisation (Jones, 1998). Cytostatic activity responsible for meiotic arrest has been shown in mammals. Oocytes of c-mos$^{-/-}$ mice undergo spontaneous activation after ovulation (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). Two recent studies using double-stranded RNA interference confirm the role of Mos on meiotic arrest in mammals (Wianny and Zernicka-Goetz, 2000) and its importance in MAPk activation (Svoboda *et al.*, 2000). Two more components of the CSF, MAPk and p90^{Rsk}, are fully activated during MII arrest in mouse oocytes (Kalab *et al.*, 1996).

The effect of Ca^{2+} at fertilisation can be proved by the addition of the calcium chelator BAPTA-AM in the fertilisation medium. No cortical granule exocytosis and no completion of meiosis occur when BAPTA-AM is present (Kline and Kline, 1992). However, when a Ca^{2+} ionophore is applied, MII oocytes undergo cortical granule exocytosis, second polar body extrusion, pronuclear formation and development to blastocyst (Susko-Parrish *et al.*, 1994). The evidence presented before indicates that a Ca^{2+} rise and its subsequent oscillations that last several hours are essential to ensure the reinitiation and completion of meiosis in mammals.

Ca^{2+} mediated signal transduction

Three main hypotheses have been proposed as possible signalling pathways for sperm-induced oocyte activation. The first, the sperm conduit model, suggests a Ca^{2+} flow through a channel into the oocyte. In the free sperm, Ca^{2+} flows in the acrosomal process. When sperm and oocyte fuse, a steady flow of Ca^{2+} is pumped into the endoplasmic reticulum of the oocyte. This finally overloads to start the fertilisation wave (Jaffe, 1983; Creton and Jaffe, 1995). However, evidence argues against the sperm conduit model in mammals. As shown by Jones *et al.* (1998b), no increase in the Ca^{2+} concentration is observed near the site of sperm-egg fusion. Moreover, when decreasing the levels of extracellular Ca^{2+} to levels that should prevent Ca^{2+} flow from the sperm, the activation of the oocyte is not inhibited (Jones *et al.*, 1998b).

The second hypothesis proposes a receptor-mediated signal transduction localised on the oocyte plasma membrane. The activated membrane receptor binds to a G-protein or a PTK and activates PLC that induces the hydrolysis of PIP_2 into diacylglycerol and IP_3 (Fig. 1). Several studies support this theory in sea urchin, *Xenopus laevis* (reviewed by Sato *et al.*, 2000) and mammals

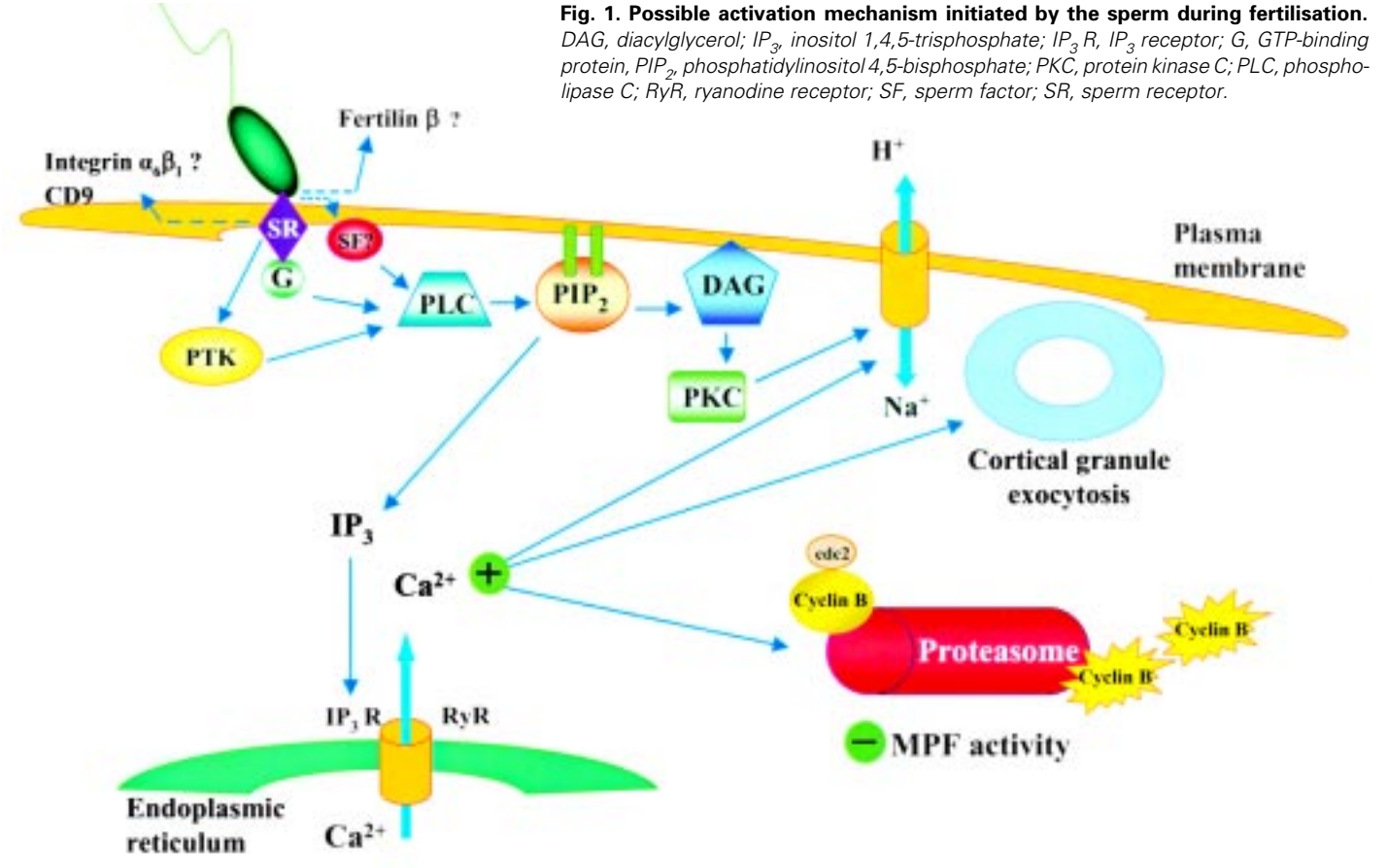


Fig. 1. Possible activation mechanism initiated by the sperm during fertilisation. *DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; G, GTP-binding protein, PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; RyR, ryanodine receptor; SF, sperm factor; SR, sperm receptor.*

(Miyazaki, 1988; Fissore *et al.*, 1995), however, no specific receptors have been identified on the oocyte plasma membrane. It has been proposed that integrins, which are present on the surface of mouse oocytes, could be involved in the sperm-oocyte binding process (Almeida *et al.*, 1995), but a direct relation to sperm-oocyte binding remains to be determined. Recently, the egg surface protein CD9 has been shown to be essential in sperm-egg fusion (Chen *et al.*, 1999; Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000). Indeed, it is proposed that the interaction of CD9 with the integrin $\alpha_6\beta_1$ could affect the ability of this integrin to bind to the sperm ligand (Chen *et al.*, 1999; Kaji *et al.*, 2000; Le Naour *et al.*, 2000). Miyado and colleagues (2000) propose alternatively that the $\alpha_6\beta_1$ integrin transduces signals to CD9 to initiate/promote fusion. However, a recent study demonstrates that the $\alpha_6\beta_1$ integrin is not essential for sperm-egg fusion and CD9 acts by itself or by interacting with egg proteins other than the $\alpha_6\beta_1$ integrin during the sperm-egg fusion in the mouse (Miller *et al.*, 2000). The sperm ligand is still unknown, however, the cell surface protein present in mouse sperms, fertilin β, is necessary for sperm-egg fusion (Cho *et al.*, 1998). While fertilin β is necessary for sperm-egg fusion, absence of this protein in sperms and eggs does not interfere with normal Ca^{2+} oscillations and egg activation (Cho *et al.*, 1998).

After sperm-egg fusion the activated receptor may bind to a G protein or PTK as a part of the signalling cascade (Fig. 1). However, a recent report suggests that nitric oxide, produced by a sperm and/or egg nitric oxide synthase, is the universal trigger of egg activation by increasing intracellular calcium release (Kuo *et al.*, 2000). The nitric oxide mediated calcium rise may involve activation of Src-like kinase and subsequently of PLCγ (Hyslop *et al.*, 2001). Though this might be possible in sea urchin eggs, a recent study demonstrates that no increase in nitric oxide is detected during fertilisation in ascidian and mouse oocytes, arguing against a specific role of nitric oxide during fertilisation in these species (Hyslop *et al.*, 2001).

Fertilisation-induced PTK phosphorylation has been observed in sea urchins, starfish, ascidians and frogs. Phosphorylation of Xyk, a tyrosine kinase purified from *Xenopus laevis* (Sato *et al.*, 1996), is not observed when either electrical shock or calcium ionophore treatment are applied, indicating that elevation of intracellular calcium is not sufficient to initiate activation and translocation of Xyk. This suggests that sperm-egg interaction leads to the activation of PTK upstream of calcium signalling during *Xenopus* fertilisation (Sato *et al.*, 2000). Tyrosine phosphorylation during rat fertilisation has been reported by Ben-Yosef and co-workers (1998), and a significant inhibition of mouse fertilisation is observed when PTKs are inhibited (Dupont *et al.*, 1996). In accordance to this, inhibition of PTK inhibits parthenogenetic activation in MII porcine oocytes, suggesting that PTKs are involved in oocyte activation in mammals (Kim *et al.*, 1999). Inhibition of protein tyrosine phosphatases by sodium orthovanadate induces high pronuclear formation, cortical granule exocytosis, and a decrease in MAPk activity (Kim *et al.*, 1999). These events are inhibited when

oocytes are pre-incubated with the calcium chelator BAPTA-AM, indicating that the stimulation of tyrosine kinase is responsible for a calcium-dependent signalling pathway that leads to the activation events associated with fertilisation (Kim *et al.*, 1999).

According to the receptor–mediated hypothesis of fertilisation, stimulation of membrane receptors initiates the phosphoinositide pathway via G-protein. The effect of G-protein in fertilisation has also been studied in mammals. Injection of a hydrolysable form of G-protein in hamster oocytes induces parthenogenetic activation (Miyazaki, 1988). In contrast, inhibition of G-protein function inhibits sperm-induced calcium release and oocyte activation (Moore *et al.*, 1993), suggesting that G-protein activation is upstream of calcium release. Although several G-proteins have been studied it is still unknown which type of G-protein is involved in mammalian egg activation (for review see Sato *et al.*, 2000). Injection of guanosine-5'-O-(3'-thiotriphosphate) (GTP-gamma-S), a hydrolysis-resistant analogue of guanosine triphosphate (GTP) that activates G-proteins, induces parthenogenetic activation in hamster and pig oocytes (Miyazaki, 1988; Macháty *et al.*, 1995). Experiments in mice and pigs demonstrate that overexpression of membrane receptors that bind to G-protein and activate phospholipase C lead to calcium oscillations and oocyte activation (Moore *et al.*, 1993; Macháty *et al.*, 1997a). In contrast, when G-proteins are inhibited no oocyte activation occurs in the absence of sperms (Moore *et al.*, 1994). Stimulating surface integrins via an RGD-peptide induces calcium transients and parthenogenetic activation (Campbell *et al.*, 2000), suggesting that G-protein-mediated signal transduction pathways exist in bovine oocytes.

The receptor-mediated hypothesis involves the activation of phospholipase C that catalyses the hydrolysis of PIP_2 in the plasma membrane generating IP_3 and diacylglycerol. IP_3 binds to its receptor present on the surface of the endoplasmic reticulum and elicits the transit of Ca^{2+} into the cytoplasm (Berridge, 1993; Fig. 1). An inositol 1,4,5-trisphosphate-induced Ca^{2+}-release demonstrated in hamster eggs is responsible for the oscillations in intracellular calcium after fertilisation (Miyazaki *et al.*, 1992b). Brind and colleagues (2000) demonstrated that IP_3R-depleted mouse oocytes do not show the sperm-induced calcium signalling observed in control oocytes indicating that Ca^{2+}-signalling at fertilisation is mediated via the IP_3R in mouse. This is further demonstrated by the fact that down-regulation of IP_3R is not induced by $SrCl_2$, suggesting that persistent stimulation of the phosphoinositide pathway in mouse oocytes by the sperm during fertilisation or by injection of SE leads to down-regulation of the type 1 IP_3R (Jallerette *et al.*, 2000). Indeed, type 1 IP_3R is the predominant isoform present in mouse and bovine MII oocytes (Parrington *et al.*, 1998; He *et al.*, 1999). Down-regulation of IP_3 receptors has been observed in mouse (Parrington *et al.*, 1998) and bovine (He *et al.*, 1999) oocytes after fertilisation, indicating that the decrease in the amplitude of sperm-induced intracellular calcium oscillations as fertilisation progresses toward pronuclear formation is a consequence of down-regulation of the second messenger IP_3. In contrast, sperm-induced IP_3R down-regulation is not observed when mouse oocytes are parthenogenetically activated (Brind *et al.*, 2000; Jallerette *et al.*, 2000).

Calcium release from RyRs induces intracellular Ca^{2+} oscillations in mouse oocytes (Swann, 1992). However, the results are contradictory since another study reports no calcium transients after injection of ryanodine into mouse MII oocytes (Kline and Kline, 1994). The authors suggested that differences might arise due to different mouse strains used in this study (Kline and Kline, 1994). Hamster oocytes do not possess RyRs, and no calcium rise is induced after injection of ryanodine (Miyazaki *et al.*, 1992b). In contrast, RyRs have been localised in the cortex of mouse (Ayabe *et al.*, 1995) and bovine (Yue *et al.*, 1998) MII oocytes in a region apposed to the meiotic spindle (Ayabe *et al.*, 1995). Specific inhibition of RyRs or IP_3Rs does not interfere reciprocally with their ability to convert the zona pellucida glycoprotein ZP2 into ZP2f, a post-fertilisation form (Ayabe *et al.*, 1995). Moreover, when RyRs are inhibited there is no interference with the activation following fertilisation, suggesting that although RyRs are present and functional, release of Ca^{2+} from this store is not essential for sperm-induced egg activation (Ayabe *et al.*, 1995). A co-operative effect of ryanodine-sensitive and ryanodine-insensitive calcium stores has been suggested in maintaining sperm-induced Ca^{2+}-oscillations in human oocytes (Sousa *et al.*, 1996). Injection of ryanodine induces calcium transients (Fissore *et al.*, 1995), pronuclear formation and cleavage in bovine oocytes, supporting the idea that this receptor may play a role in fertilisation of bovine oocytes (Yue *et al.*, 1998).

The third hypothesis of Ca^{2+}-mediated signal transduction suggests the introduction by the sperm of a soluble cytosolic factor that triggers Ca^{2+} release (Dale *et al.*, 1985; Swann, 1990). Several studies have been carried out in marine invertebrates showing the effect of SE in oocytes. When SE is injected into acidian oocytes the Ca^{2+} oscillations observed are similar to those registered after fertilisation. A spatio-temporal study of Ca^{2+} release suggests a higher sensitivity of the oocyte cortex to sperm factor (Kyozuka *et al.*, 1998). Moreover, SE-induced Ca^{2+} oscillations are dependent on oocyte CDK activity (McDougall *et al.*, 2000). A recent study shows a similar signalling pathway in response to SE injection in ascidians as observed at fertilisation supporting the hypothesis that a soluble sperm factor initiates Ca^{2+} release at fertilisation (Runft and Jaffe, 2000). Ca^{2+} oscillations similar to those observed after fertilisation are observed after injection of SE into hamster (Swann, 1990), mouse (Swann, 1994), bovine (Wu *et al.*, 1997) and human oocytes (Homa and Swann, 1994). Furthermore, oocyte activation and development to blastocyst have been reported in bovine oocytes after SE injection (Fissore *et al.*, 1998). Cross-reactivity between sea urchin SE and mouse oocytes, and porcine SE and oocytes of a marine worm, suggests that the signalling molecule(s) present in the extracts is(are) well conserved among species (Parrington *et al.*, 1999; Stricker *et al.*, 2000). The nature of this factor has not been identified, however, it has been determined that it is thermolabile (Kyozuka *et al.*, 1998). Moreover, different activity is detected in fractions of SE suggesting a single protein factor as the active component of the SE in ascidians (McDougall *et al.*, 2000). As recently demonstrated, sperm factor is able to induce increases in IP_3 in sea urchin egg homogenates (Jones *et al.*, 2000) and mouse oocytes (Wu *et al.*, 2001). This suggests that sperm factor initiates and sustains calcium rises by activating the phosphoinositide pathway (Wu *et al.*, 2001).

PLCγ1, PLCγ2 and PLCδ4 have been identified in mouse sperms (PLCγ1 and PLCγ2) and rat testis (PLCδ4) (Dupont *et al.*, 1996; Lee and Rhee, 1996; Mehlmann *et al.*, 1998). A PLC activity present in SE induces Ca^{2+} oscillations in sea urchin extracts (Jones *et al.*, 1998b), suggesting that the sperm factor is a PLC

(Swann and Parrington, 1999). Rice and colleagues (2000) showed that a sperm cytosolic Ca^{2+}-sensitive PLC activity generates IP_3 from the hydrolysis of PIP_2 present in intracellular membranes of sea urchin egg homogenates. In an attempt to identify the sperm PLC, the ability to generate IP_3 and calcium release from SE, different tissue-homogenates (as other sources of PLCs) and recombinant PLCs (with greater specific activity) have been compared (Jones *et al.*, 2000; Rice *et al.*, 2000). Since no PLC activity has been found in sea urchin homogenates and mouse oocytes when injected either with extracts from different tissues and with recombinant PLCs, it is suggested that a novel type of PLC or a specifically regulated PLC is present in sperms (Jones *et al.*, 2000). Two recent reports show that PLCγ1, PLCγ2 and PLCδ4 present in fractions of boar SE do not induce calcium release and oocyte activation in mouse-injected oocytes (Heyers *et al.*, 2000; Wu *et al.*, 2001), confirming observations in sea urchin egg homogenates (Jones *et al.*, 2000). A recent study shows that PLCδ4$^{-/-}$ mouse sperms fail to fertilize wild-type oocytes, demonstrating that PLCδ4 plays an essential role in the zona-pellucida induced acrosome reaction (Fukami *et al.*, 2001). However, PLCδ4$^{-/-}$ and PLCδ4$^{+/+}$ sperms injected into eggs induce similar calcium transients and activation rates, indicating that PLCδ4 is essential for events preceding or leading to sperm fusion (Fukami *et al.*, 2001).

A protein of 33 kDa named "oscillin" has been proposed as the oscillogenic factor, since it induces Ca^{2+} oscillations in mouse oocytes (Parrington *et al.*, 1996). However, it has recently been demonstrated that a recombinant oscillin does not trigger Ca^{2+} oscillations when injected into oocytes (Shevchenko *et al.*, 1998). Other sperm proteins have been suggested to be Ca^{2+}-activating proteins like the protein tyrosine kinase truncated c-kit (Sette *et al.*, 1997) and a perinuclear protein (Kimura *et al.*, 1998), which could be part of the perinuclear theca (Oko and Maravei, 1994). The truncated c-kit gene product is absent in round spermatids and accumulates in elongated spermatids. The fact that injection of round spermatids does not activate mouse oocytes (Kimura and Yanagimachi, 1995) suggests that the truncated c-kit might be an important protein involved in sperm-induced oocyte activation. Moreover, it has been shown that truncated c-kit-mediated egg activation involves activation of PLCγ (Sette *et al.*, 1998). Though the presence of a cytosolic factor is supported by several findings, a recent report by Perry *et al.* (2000) demonstrates the presence of at least two submembrane components with activating activity named SOAF. SOAF can be divided into a heat-sensitive and a stable component. When injected individually both components fail to induce calcium oscillations, while when co-injected the oscillations resemble those initiated by sperms and induce oocyte activation. This is the first evidence that multiple components are involved in the induction of calcium oscillations (Perry *et al.*, 2000). The SOAF components are liberated from the perinuclear matrix by an endoprotease that becomes activated upon exposure to the reducing environment of the oocyte (Calvin *et al.*, 1986). The SOAF may be part of the perinuclear theca as described by Oko and Maravei (1994). The perinuclear theca is the cytoskeletal coat of the sperm nucleus composed of myelin and cytokeratin-like proteins (Sutovsky and Schatten, 2000). Numerous proteins have been identified in the perinuclear theca of mouse, hamster, rat, pig, cattle and human sperms (reviewed by Sutovsky and Schatten, 2000). Moreover, sperm from patients with spermatogenetic disorders associated with the absence of perinuclear theca fail to activate human oocytes after intracytoplasmic sperm injection (Battaglia *et al.*, 1997). This suggests that components of the perinuclear theca are involved in the activation signals during fertilisation. The conclusion of Perry and co-workers (2000) proposing multiple components as part of SOAF contrasts with the hypothesis of a single cytosolic (probably a PLC) sperm factor proposed by several authors (Swann and Parrington, 1999; Jones *et al.*, 2000; Rice *et al.*, 2000). Further studies are certainly needed to elucidate the nature of SOAF.

While a sperm-mediated signal is generally accepted, little is known about the oocyte response to the sperm-induced activation. A recent study proposes that the ability of an oocyte to respond to sperm-induced calcium oscillations is dependent on the presence of a machinery in the oocyte that is functional only once in mammalian oocytes, and is inactivated by sperm components but not after parthenogenetic activation (Tang *et al.*, 2000). The inactivation of this maternal machinery is neither dependent on IP_3 receptor sensitivity nor on the calcium content of the oocyte (Tang *et al.*, 2000).

Sperm-induced calcium oscillations initiate a series of biochemical events leading to full activation of the oocyte. This is characterised by resumption of meiosis, pronuclear formation and DNA replication. Finally, pronuclear apposition occurs, the nuclear envelope breaks down and the chromatin condenses into chromosomes. The condensed chromosomes arrange themselves on a common mitotic spindle and they are ready for the first cleavage division. Thus, no pronuclear fusion occurs in mammals (*Ascaris* type of fertilisation) as it is observed in sea urchins (Longo, 1997). Moreover, a recent study shows that chromosomes stay separate according to parental origin up to the 4-cell stage in the mouse. As demonstrated by Mayer *et al.* (2000), when sperm-derived chromosomes were stained with BrdU a topological separation of the parental genomes is observed suggesting that parental chromatin is not randomly distributed in the preimplantation embryo.

In the following part of the review the biochemical changes occurring after fertilisation are compared to those observed after artificial oocyte activation.

Artificial oocyte activation

As described before, the sperm is the natural stimulus responsible for the activation of matured oocytes. However, certain artificial stimuli trigger oocyte activation and elicit development to blastocyst. The development to blastocyst of a female gamete without contribution of a male gamete is defined as parthenogenesis (Kaufman, 1979). Parthenogenesis has been used as a model to study biochemical and morphological events occurring during early embryonic development (Collas *et al.*, 1993). It has also important implications for the successful performance of biotechniques like NT.

Stimulating calcium signalling

Since Ca^{2+} transient is the key trigger of meiotic resumption during fertilisation a wide range of procedures for artificial oocyte activation have been established including mechanical, chemical and physical stimuli that elicit one or several Ca^{2+} transients in the oocyte. Mechanical disruption of the plasma membrane of frog oocytes with a fine needle is sufficient to generate Ca^{2+} influx and

to initiate development (Kawamura, 1939). Microinjection of Ca^{2+} is another way to increase intracellular calcium. This is effective for the activation of pig oocytes where all the events normally occurring following fertilisation, i.e. cortical granule exocytosis, decrease of the H1 kinase activity (as an indicator of MPF activity), changes in the protein synthetic profile, and pronuclear formation, are observed after microinjection of $CaCl_2$ (Macháty *et al.*, 1996).

Chemical activation can be induced by exposure to Ca^{2+} ionophore, 7% ethanol, strontium chloride, phorbol ester and thimerosal (Nakada and Mizuno, 1998; reviewed by Macháty *et al.,* 1998). Ionophore A23187 promotes the release of intracellular Ca^{2+} stores but also facilitates the influx of extracellular Ca^{2+} ions (Kline and Kline, 1992). Ionomycin is another potent Ca^{2+} ionophore currently used in NT protocols (Cibelli *et al.*, 1998; Wells *et al.*, 1999). It mobilises intracellular Ca^{2+} by depletion of Ca^{2+} stores. Exposure of matured oocytes to 7% ethanol for 5-7 min induces successful activation and pronuclear formation (Presicce and Yang, 1994) by promoting the formation of IP_3 and the influx of extracellular Ca^{2+}. The substances mentioned above induce a single Ca^{2+} rise in the oocyte. However, the initial Ca^{2+} rise is normally followed by Ca^{2+} oscillations during fertilisation in mammals. Strontium chloride induces multiple Ca^{2+} transients probably by displacing bound Ca^{2+} in the oocyte (Whittingham and Siracussa, 1978) but also by inducing intracellular Ca^{2+} release (Kline and Kline, 1992). Strontium chloride has been successfully used to activate mouse oocytes after NT (Wakayama *et al.*, 1998). Phorbol ester, which mimics endogenous diacylglycerol, activates the calcium- and phospholipid-dependent protein kinase C (Nishizuka *et al.*, 1984) and induces calcium oscillations and pronuclear formation in mouse oocytes (Cuthbertson and Cobbold, 1985). However, activation rate is lower when compared to calcium ionophore (Uranga *et al.*, 1996). This compound has not been used so far in other mammalian oocytes. Thimerosal, a sulfhydryl-oxidising agent that induces repetitive Ca^{2+} oscillations, has been successfully used for the activation of bovine oocytes (Fissore *et al.*, 1992; 1995). However, the peak and the duration of the calcium oscillations induced by thimerosal are shorter than those of the first rise induced by spermatozoa during fertilisation (Nakada and Mizuno, 1998). While mouse oocytes show repetitive calcium transients when incubated with thimerosal (Cheek *et al.*, 1993; Kline and Kline, 1994) no oocyte activation is observed (Cheek *et al.*, 1993). Evidence of destruction of the meiotic spindle has been detected in thimerosal-incubated oocytes after staining of tubulin and chromatin (Cheek *et al.*, 1993). Thimerosal is acting on IP_3-induced Ca^{2+} release in mouse oocytes (Kline and Kline, 1994). In accordance to this report, repetitive calcium oscillations are observed in thimerosal-treated hamster (Swann, 1991; Miyazaki *et al.*, 1992a) and rabbit oocytes (Fissore and Robl, 1993). This is mediated by IP_3 release (Miyazaki *et al.*, 1992a) and activation of IP_3R (Fissore and Robl, 1993). Thimerosal incubation followed by it inhibitor dithiothreitol induces parthenogenetic activation and successful development to the blastocyst of pig oocytes (Macháty *et al.*, 1997b). When used after reconstructing embryos by NT thimerosal/dithiothreitol induces similar pronuclear formation rate to those obtained after electrical stimulation (Tao *et al.*, 2000).

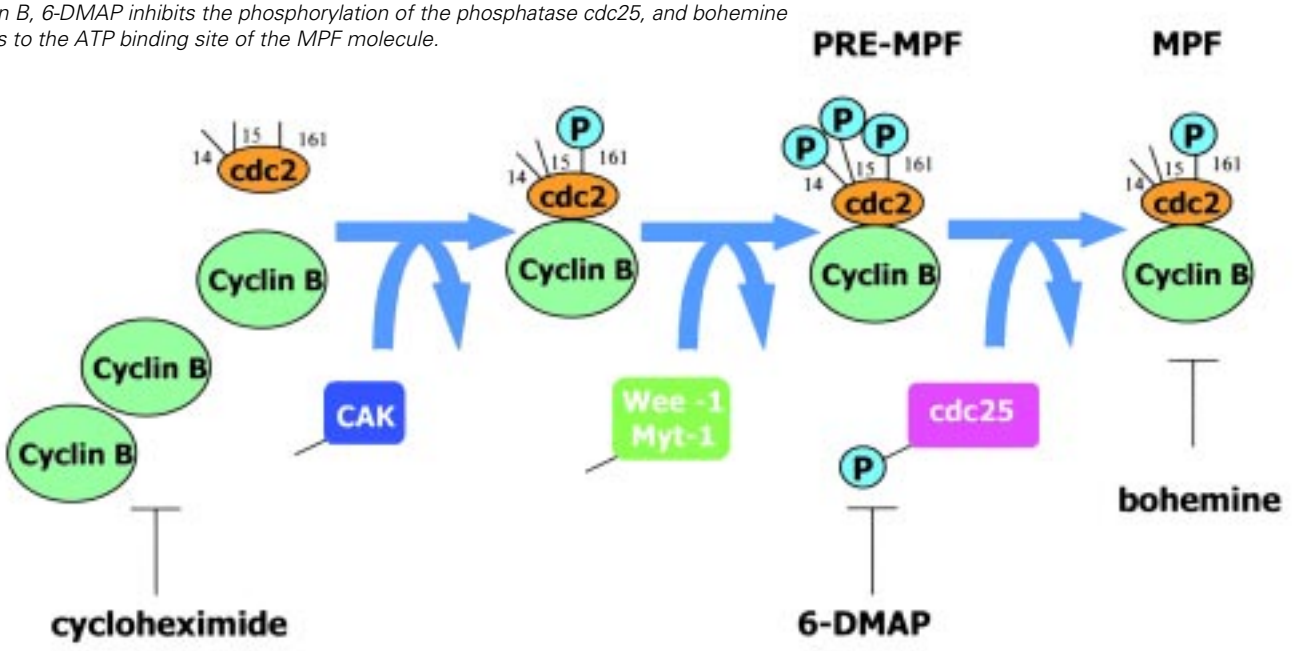
Electrical stimulation is an alternative to chemical activation to induce Ca^{2+} influx through the formation of pores in the plasma membrane. The success of this procedure depends on the size of the pores formed but also on the ionic content of the medium and the cell type. Moreover, the time to restore the membrane integrity is dependent on the temperature that should ensure the fluidity of lipids and proteins in the membranes (Zimmermann *et al.*, 1985). Periodically repeated electrical stimulation mimics the pattern of oscillations observed during fertilisation (Ozil, 1990). The single Ca^{2+} rise recorded after electrical stimulation is dependent on the presence of extracellular Ca^{2+} ions. However, when rabbit oocytes are pulsed in the presence of lithium (which prevents the production of IP_3) oocyte activation is inhibited (Ozil, 1990). This suggests that electrical stimulation induces the production of IP_3 that leads to intracellular Ca^{2+} release. Electroporation of IP_3 in a calcium- and magnesium-free medium followed by incubation in 6-DMAP has been used to activate parthenogenetic and NT rabbit embryos (Mitalipov *et al.*, 1999). Another physical stimulus used for oocyte activation is the exposure of oocytes to room temperature prior NT (Stice *et al.*, 1994).

Inhibiting MPF and MAPk activity

The drop of MPF and MAPk activity should be triggered to induce resumption of meiosis, chromatin decondensation and transition to interphase. MPF has been described as the biological activity of cytoplasm capable of reinitiating meiosis in prophase arrested oocytes (Masui and Markert, 1971) and is essential for meiotic arrest at MII (Nurse, 1990). Since the concentration of cyclin B oscillates during the cell cycle, the level of MPF molecules depends on the synthesis and degradation of cyclin B. MPF activity rises during oocyte maturation and the phosphorylation status of its constituents determines its kinase activity (Motlik *et al.*, 1998). This phosphorylation is regulated by specific kinases like cyclin-activating kinase, and other kinases like Myt-1 and Wee-1 (described in *Xenopus laevis* and *Schizosaccharomyces pombe*). The phosphatase cdc25 activates MPF by dephosphorylation of the tyr15 and thr14 sites of cdc2 (Fig. 2). Specific substances that inhibit the activation site of this phosphatase are currently used for oocyte activation (see below). During the MII arrest the high MPF activity is maintained through continuous equilibrium between cyclin B degradation and synthesis (Kubiak *et al.*, 1993). This explains why protein synthesis inhibitors effectively induce oocyte activation (see below). MPF activity is stabilised by CSF, which consists of Mos, MAPk, and $p90^{Rsk}$. These proteins maintain the condensed status of chromatin, thus avoiding DNA replication between MI and MII (Verlhac *et al.*, 1994). The pattern of inactivation of MPF and MAPk activities after activation is used as indicator for the success and efficacy of artificial activation protocols.

One possible way to inhibit MPF is to incubate matured oocytes in broad-spectrum inhibitors of protein synthesis and/or protein phosphorylation (Fig. 2). Two inhibitors of protein synthesis, cycloheximide and puromycin, induce oocyte activation after prolonged periods of incubation in mouse (Moses and Kline, 1995b) and human oocytes (Balakier and Casper, 1993). However, in rat (Zernicka-Goetz *et al.*, 1993) and pig oocytes (Nussbaum and Prather, 1995) these inhibitors are not sufficient to induce activation. The combined use of a Ca^{2+} stimulating substance with an inhibitor of protein synthesis has been widely tested for activation of mouse, sheep and cattle oocytes. Ca^{2+} ionophore plus cycloheximide induce a high rate of pronuclear formation and development

Fig. 2. Different ways to inhibit MPF activity. *Cycloheximide inhibits the synthesis of cyclin B, 6-DMAP inhibits the phosphorylation of the phosphatase cdc25, and bohemine binds to the ATP binding site of the MPF molecule.*



to blastocyst in mouse oocytes (Hagemann *et al*., 1995). Similar results have been obtained with ethanol plus cycloheximide in bovine (Presicce and Yang, 1994) and when used after somatic NT several offspring have been produced (Zakhartchenko *et al*., 1999a,b). However, as stated by Soloy *et al*. (1997), cycloheximide not only depletes the oocyte from proteins maintaining MPF activity, but also inhibits the translation of proteins responsible for the initiation of DNA replication. Indeed, initiation of DNA synthesis is delayed in NT embryos activated by ethanol plus cycloheximide (Alberio *et al*., 2001). Hence, postimplantation development is affected in a high proportion of NT embryos produced with cycloheximide showing pregnancy loss, hydroallantois, perinatal death and skeletal malformations (Zakhartchenko *et al*., 1999a,b and unpublished observations). Whether the delayed cleavage due to this activation procedure affects postimplantation development is unclear, however, early embryonic cleavage is not random and it has important consequences in further developmental events, i.e. establishment of cell lineages and cell differentiation (Piotrowska and Zernicka-Goetz, 2001).

A more specific inhibition can be achieved by inhibition of protein kinases. It has been shown that a cAMP-dependent kinase inhibitor localises in the nucleus of G2/M cells. When inhibited, the cell cycle is arrested suggesting that protein kinase inhibition may be a normal function for the transition from M-phase to G1 (Wen *et al*., 1995). When *Xenopus laevis* oocytes are exposed to 6-DMAP, a phosphatase inhibitor, resumption of meiosis without Ca^{2+} release is observed (Zhang and Masui, 1992). Similarly, pig oocytes treated with staurosporine and H7 (both inhibitors of protein kinases) resume meiosis without Ca^{2+} release (Rickords *et al*., 1992), and undergo pronuclear formation and development to blastocyst (Wang *et al*., 1997). However, mouse and bovine oocytes are not activated when incubated in 6-DMAP without previous Ca^{2+} release (Szöllösi *et al*., 1993; Liu *et al*., 1998).

The combination of a Ca^{2+} ionophore with 6-DMAP induces high rates of activation, pronuclear formation and development to blastocyst in ovine (Loi *et al*., 1998) and bovine (Liu *et al*., 1998). These protocols have been successfully used for the production of cloned calves after somatic NT (Cibelli *et al*., 1998). However, oocytes activated with ionomycin and 6-DMAP display some alterations in the DNA content, reflecting an abnormal pattern of karyokinesis during the first cell cycle (de la Fuente and King, 1998). Numerous failures in the establishment of pregnancies, placental malformations and perinatal death have been reported after activation of NT embryos with this protocol (Cibelli *et al*., 1998). 6-DMAP inhibits phosphorylation of ribosomal protein S6 and activation of the 70-kDa S6 kinase in somatic cells and it drastically affects cytoskeletal components leading to the formation of micronuclei containing chromosomes. This suggests that a disturbance in G1 of a signal transduction pathway may contribute to abnormal mitosis (Simily *et al*., 1997). As shown by Schlegel *et al*. (1990), 6-DMAP induces premature chromatin condensation and premature mitosis in cells arrested in S-phase, suggesting a role for protein dephosphorylation in the control of mitosis (Schlegel *et al*., 1990). Protein kinase inhibition is an efficient way to induce oocyte activation, however, it should be considered that these inhibitors are not specifically interfering with one kinase, but with several involved in other cell functions, whose inhibition may be deleterious in subsequent cellular events after activation.

MAPk is the family name for a number of Ser/Thr protein kinases. Two MAPk isozymes are active during meiosis: the extracellular signal-regulated protein kinases 1 and 2 (Verlhac *et al*., 1994). Phosphorylated MAPk localises in the spindle poles of mouse MII oocytes, suggesting that this protein plays a role on spindle organisation (Verlhac *et al*., 1993). Oocytes from *mos* knockout mice, lacking MAPk activity, show diffuse spindles and are unable to extrude a normal polar body (Choi *et al*., 1996). Further evidence of the significance of MAPk activity for spindle organisation has been provided in porcine and bovine oocytes. When MAPk activity is inhibited, disorganisation of meiotic spindle and impaired polar body extrusion are observed in bovine and

porcine activated oocytes, respectively (Gordo *et al.*, 2001; Tatemoto and Muto, 2001). MAPk activation is essential for MII arrest in mouse (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994) and bovine oocytes (Gordo *et al.*, 2001). The decrease in MAPk activity is correlated with the formation of a nuclear envelope after parthenogenetic activation of mouse (Moos *et al.*, 1995) and cattle oocytes (Liu and Yang, 1999). Moreover, MAPk inactivation correlates with the initiation of DNA synthesis (Carroll *et al.*, 2000). This suggests that inhibition of MAPk activity, independently of MPF inactivation, leads to oocyte activation. Indeed, it has been shown that pig oocytes incubated with the MAPk inhibitor U0126 do complete meiosis and form a pronucleus (24%), however, polar body emission is impaired (Tatemoto and Muto, 2001). Moreover, while MAPk inactivation is accelerated in U0126-treated oocytes, there is no difference in the time course of pronuclear formation, suggesting that MAPk activity is partly involved in pronuclear development (Tatemoto and Muto, 2001). Inactivation of MAPk can also be induced by stimulation of protein kinase C. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, induces activation of mouse oocytes, and inactivation of MAP kinase (Sun *et al.*, 1999). The PMA-mediated activation is not dependent on calcium increase (Moses and Kline, 1995a).

Protein phosphatase inhibitors have also been tested to induce oocyte activation. When pig oocytes were activated by ionomycin and subsequently incubated in okadaic acid, an inhibitor of protein phosphatases 1 and 2A, no oocyte activation has been observed (Grocholova *et al.*, 1997). In rats, incubation of puromycin-activated oocytes with okadaic acid inhibits parthenogenetic activation as well (Zernicka-Goetz *et al.*, 1993). Mouse oocytes activated with A23187 and okadaic acid show disruption of the spindle, disjunction of chromosomes and no pronuclear formation; however, when this treatment is combined with 6-DMAP, pronuclear formation is observed (Moses, 1996). Protein phosphatase activity determined in activated oocytes demonstrates that the type 2A protein phosphatase plays a role in cell cycle regulation and undergoes changes in its activity during early mammalian development (Winston and Maro, 1999).

Multiple factors may be involved in the alterations observed in NT embryos, however, the activation stimulus should not be neglected as a possible cause of failure in the production of normal cloned animals. Non-specific inhibition of several metabolic pathways in oocytes may affect the selective activation/inhibition of specific kinases and phosphatases and may have negative consequences for embryonic development.

Cyclin-dependent kinase inhibitors

Since a co-ordinated series of events is responsible for the signalling pathway initiated by the sperm during fertilisation, it is reasonable to think that specific kinases and phosphatases are involved differentially in the transition from MII arrest into interphase. The cell cycle is regulated by kinases that are activated by cyclin binding and phosphorylation, and inhibited by phosphorylation, proteolysis and by binding of specific CDKIs. Natural CDKIs play a major role in the control of the cell cycle progression. By forming quaternary complexes with the target kinase, the CDKI $p21^{cip1}$ induces G1 arrest and its expression correlates with terminal differentiation in several cell lineages (Jiang *et al.*, 1994). Several CDKIs have been described as mediating extracellular negative signals that result in G1 arrest by specific cyclin/cdk complex inhibition. Moreover, they have also been implicated in the mechanisms of checkpoint control of DNA integrity and spindle formation (Graña and Reddy, 1995). The development of synthetic CDKIs in the last years has been continuously growing, since several human diseases can be treated with these compounds. The first compound identified as CDKI has been 6-DMAP, a substance of low selectivity. Butyrolactone I, isolated from Aspergillus strain 25799, inhibits selectively cdk2 and cdc2, arresting the cell cycle at G2/M and G1/S (Kitagawa *et al.*, 1993; Gray *et al.*, 1999). By using combinatorial chemistry new compounds have been synthesised with high selectivity and efficiency. These purine analogues target the ATP-binding site of cyclin/cdk molecules (Kitagawa *et al.*, 1993). Olomoucine, roscovitine and bohemine are some of these new compounds with higher selectivity than butyrolactone I (Gray *et al.*, 1999; Hájduch *et al.*, 1999). The inhibition of specific kinases by these molecules during oocyte activation is of high interest in a way to mimic sperm-mediated events during fertilisation.

Targeted oocyte activation

The use of this new generation of CDKIs for inhibition of MPF activity has been proposed by Motlik *et al.* (1998). Since then, several studies have been carried out to evaluate the effects of these drugs on cell cycle progression, oocyte maturation and oocyte activation. The high reversibility upon cell cycle progression in somatic cells (Alessi *et al.*, 1998) and development to embryos of bovine oocytes artificially arrested by these inhibitors before *in vitro* maturation and fertilisation (Mermillod *et al.*, 2000) suggest that these substances do not impair the viability of treated cells. Moreover, when MII arrested bovine oocytes are activated with bohemine for 6 hours high pronuclear formation rate is observed (> 95%) and DNA synthesis starts synchronously in more than 85% of the oocytes 2 hours after withdrawal from the inhibitor (Alberio *et al.*, 2000). A recent study also shows that, when bovine and porcine oocytes are specifically targeted with butyrolactone I, the MPF activity is inhibited, while the condensing activity of chromosomes is not affected (Kubelka *et al.*, 2000). In contrast, fertilisation-induced calcium oscillations are inhibited in mouse oocytes fertilised in the presence of roscovitine (Deng and Shen, 2000).

The early initiation of DNA synthetic activity after removal from the activation medium (Alberio *et al.,* 2000), the maintained ability to condense chromatin (Kubelka *et al.*, 2000) and the high development to blastocysts in bovine (Mermillod *et al.*, 2000, Lonergan *et al.*, 2000) demonstrate that these new CDKIs do not irreversibly interfere with cell cycle progression. Biochemical studies reveal that MPF activity decreases abruptly after incubation of bovine oocytes in bohemine, however, MAPk inactivation decreases more slowly (Alberio *et al.*, 2000). A similar pattern of MPF and MAPk inactivation has been observed after *in vitro* fertilisation of bovine oocytes (Liu and Yang, 1999). Chromatin remodelling after somatic NT has been evaluated in cattle after activation with bohemine. The dynamic of pronuclear development and initiation of DNA synthesis is similar as in bovine parthenotes. Moreover, after the end of the first cell cycle more than 50% of the cleaved embryos are of normal ploidy. This rate is similar to embryos activated by a standard activation protocol (Alberio *et al.*, 2001). *In vivo* data provided by Hill *et al.* (2000) demonstrate that bovine cloned

embryos activated by butyrolactone I come to birth. However, there is no improvement in the pregnancy and survival rates after birth compared to reports where other activation procedures have been used (Cibelli *et al.*, 1998; Zakhartchenko *et al.*, 1999a,b).

The studies presented above suggest that specific inhibitors of CDKs might be useful for the understanding of cellular events and may contribute to elucidate the mechanism involved in parthenogenetic or sperm-mediated oocyte activation in mammals, and consequently it may be helpful for the development of efficient activation protocols.

Summary

Events after fertilisation have been carefully studied in the last decades. However, there are still several questions to be clarified in relation to the signalling pathway initiated by the sperm, the identification of proteins or factors involved in the activation of the arrested oocyte, and the inactivation of specific molecules involved in the meiotic arrest. The present state of knowledge in mammalian fertilisation allows the development of activation protocols that closely mimic the events initiated by the sperm according to certain major factors (MPF activity and MAPk activity). These protocols are successfully used for the activation of oocytes after NT giving rise to offspring. Few cloned animals have yet been produced. However, the pregnancy and the survival rates after birth are not significantly different when different activation protocols are compared. This fact argues for a major reason for the low success in the efficiency of NT. Eventually, factors related to the recipient oocyte, the donor cell or the culture conditions are part of these major problems that the reconstructed embryo has to overcome to develop into a normal offspring. Nonetheless, the development of activation protocols that closely imitate the mechanism of activation initiated by the sperm are of special interest to improve the developmental potential of cloned embryos.

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