Development 121, 1123-1128 (1995)
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Nuclei from fertilized mouse embryos have calcium-releasing activity

Tomohiro Kono[1,2,*], John Carroll[2], Karl Swann[3] and David G. Whittingham[2]

[1]NODAI Research Institute, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan
[2]MRC Experimental Embryology and Teratology Unit, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, UK
[3]Department of Anatomy and Developmental Biology, University College, Gower Street, London WC1E 6BT, UK

*Author for correspondence at address 2

SUMMARY

During mammalian fertilization, the sperm triggers a series of intracellular Ca^{2+} oscillations which initiate oocyte activation and the formation of pronuclei. Oocyte activation can be induced artificially by a variety of chemical and physical stimuli which elevate intracellular calcium. We show that the transfer of nuclei from 1- and 2-cell-stage fertilized mouse embryos to unfertilized oocytes stimulates the completion of meiosis and the formation of pronuclei. Nuclei from embryos that had developed to the 4-cell stage did not stimulate meiotic resumption. The ability to cause oocyte activation was specific to nuclei transferred from fertilized embryos as nuclei from parthenogenetic embryos or cytoplasts from fertilized or parthenogenetic embryos did not induce activation. Nucleus-induced oocyte activation was associated with the generation of intracellular Ca^{2+} transients, which were seen after nuclear envelope breakdown of the transferred nuclei. Treatment of the oocyte with the intracellular Ca^{2+} chelator, BAPTA, prior to nuclear transfer inhibited intracellular Ca^{2+} transients and oocyte activation. The specific Ca^{2+}-releasing activity of the nucleus was not caused by sperm-induced protein synthesis since similar activity was present in nuclei originating from embryos exposed to cycloheximide throughout fertilization. The specific ability of nuclei from fertilized embryos to stimulate Ca^{2+} transients and oocyte activation was also found in nuclei from embryos parthenogenetically activated by the injection of a partially purified cytosolic sperm factor. The results suggest that the fertilizing sperm introduces Ca^{2+}-releasing activity which becomes associated with the nucleus of early mammalian embryos.

Key words: calcium, oocyte, sperm, fertilization, activation, nucleus, mouse

INTRODUCTION

In most mammalian species, the mature oocyte is arrested at metaphase of the second meiotic division (MII). The arrest at MII is maintained by high levels of maturation promoting factor (MPF) (Hashimoto and Kishimoto, 1988; Choi et al., 1991) which is stabilized by cytostatic factor (CSF) (Sagata et al., 1989; Masui, 1991). Progression beyond metaphase II requires destruction of MPF activity which is thought to be stimulated by a Ca^{2+}-mediated activation of Ca^{2+}-dependent protein kinase II (Lorca et al., 1993). Oocyte activation and cell-cycle resumption at fertilization is, therefore, triggered by an increase in the concentration of intracellular Ca^{2+} (Jaffe, 1983). In mammals the sperm initiates a series of Ca^{2+} oscillations that continue for several hours (Cuthbertson and Cobbold, 1985; Miyazaki et al., 1986; Taylor et al., 1993; Swann and Ozil, 1994). Oocyte activation can also be stimulated artificially by chemical (ethanol, Ca^{2+} ionophore, strontium) or mechanical (electric field pulse) treatments that cause an increase in Ca^{2+} (Whittingham, 1980; Cuthbertson, 1983; Ozil, 1990; Vincent et al., 1992). The increase in $[Ca^{2+}]_i$ at fertilization or parthenogenetic activation is essential since intracellular Ca^{2+} buffers inhibit the resumption of the cell cycle and cortical granule exocytosis (Kline and Kline, 1992).

It is still unclear how the sperm triggers the release of intracellular Ca^{2+} at fertilization. Two main hypotheses have been proposed. (1) The sperm binds to an oocyte receptor on the plasma membrane which is coupled to phosphoinositide turnover leading to a rise in inositol trisphosphate ($InsP_3$) and the mobilization of intracellular Ca^{2+} (Jaffe, 1990; Miyazaki et al., 1993). (2) At gamete fusion, the sperm introduces a soluble factor that stimulates the release of Ca^{2+} from intracellular Ca^{2+} stores (Dale et al., 1985; Stice and Robl, 1990; Swann, 1990). This is based on the observation that the injection of a cytosolic sperm extract stimulates Ca^{2+} oscillations and oocyte activation similar to normal fertilization (Swann, 1990).

In order to examine more closely the Ca^{2+}-releasing activity in the fertilized oocyte, we have transplanted cytoplasts and nuclei from fertilized and parthenogenetically activated embryos into unfertilized oocytes. Due to the high levels of MPF activity in mature oocytes (Hashimoto and Kishimoto, 1988; Choi et al., 1991), nuclei undergo nuclear envelope break down (NEBD), chromosome condensation and the formation of an additional metaphase spindle (Czolowska et

al., 1984; Tsunoda et al., 1989; Kono et al., 1991). In this report, we show that nuclei transferred from fertilized 1-and 2-cell embryos to unfertilized oocytes initiates Ca^{2+} oscillations and oocyte activation. This activity was not found in nuclei from parthenogenetic embryos or in cytoplasts from fertilized or parthenogenetic embryos. Finally, we show that nuclei from oocytes activated by the injection of a cytosolic sperm extract also stimulate Ca^{2+} transients and oocyte activation.

MATERIALS AND METHODS

Preparation of oocytes and embryos

B6CBF1 (C57Bl/6JLac×CBA/CaLac) and CD-1 female mice were superovulated by injections of pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) 48 hours apart. Oocytes were collected from B6CBF1 females 15-16 hours post hCG injection. Fertilized 1-, 2- and 4-cell embryos and cells from the inner cell mass of blastocysts were obtained from homologous matings of B6CBF1 or CD-1 females. All oocytes and embryos were collected and manipulated in M2 medium (Fulton and Whittingham, 1978). When required, cumulus cells were removed by treatment with 300 units hyaluronidase/ml in M2 medium. M16 medium (Whittingham, 1971) was used for culture at 37°C in an atmosphere 5% CO_2 in air.

Parthenogenetic embryos were produced by exposure of oocytes to 7% ethanol in M2 medium for 7 minutes at room temperature (Cuthbertson, 1983) or by a 90 minute incubation in Ca^{2+}-free M16 medium supplemented with 10 mM Sr^{2+} (Kline and Kline, 1992; Bos-Mikich et al., 1995). To produce diploid parthenogenones, the oocytes were cultured for a further 4 hours in M16 medium containing 5 μg/ml cytochalasin B.

Androgenetic embryos were produced by in vitro fertilization of enucleated oocytes as described previously (Kono et al., 1993). After treatment with cytochalasin B (5 μg/ml) for 10 minutes, the metaphase II chromosomes were visualized using Nomarski optics and were removed using an enucleation pipette. The enucleated oocytes were transferred into a 100 μl drop of TYH medium (Toyoda et al., 1971) in which the sperm concentration was adjusted to $50\text{-}100\times10^4$/ml. Gynogenetic embryos were produced by removal of the male pronucleus that was identified by its location in the cytoplasm and larger size (Surani et al., 1986).

For inhibition of protein synthesis after fertilization, oocytes were incubated for 4 hours with capacitated sperm in vitro (Kono et al., 1993) in TYH medium containing 10 μg cycloheximide/ml (Siracusa et al., 1978). The fertilized embryos were transferred to M16 medium containing the same concentration of cycloheximide and maintained in this medium during all subsequent manipulations.

Chelation of intracellular calcium

Intracellular Ca^{2+} was chelated by incubation of reconstructed embryos with the acetoxymethyl ester of the Ca^{2+}-chelator 1,2-bis (2-aminophenoxy) ethane N′,N′,N′,N′-tetraacetic acid (10 μM) (BAPTA-AM, Molecular Probes) in M2 medium for 30 minutes at 37°C.

Nuclear transfer

Nuclear transfer was carried out by standard micromanipulation techniques (Tsunoda et al., 1989; Kono et al., 1991). Before transfer of nuclei the zonae pellucidae of the oocytes and embryos was slit with a glass needle. Nuclear transfer was performed in a small drop of M2 medium containing cytochalasin B (5 μg/ml) and colcemid (0.1 μg/ml). Nuclei were removed from donor embryos and transferred to the peri-vitelline space of unfertilized oocytes with inactivated Sendai virus (HVJ, 2700 haemagglutinating activity unit/ml) to induce fusion, which occurred within 20 minutes.

Microinjection

Cytosolic sperm extracts were made from ejaculated boar or epididymal hamster sperm as described previously. The extracts were partially purified using a Cibacron Blue dye affinity column (Swann, 1994). The fractions from the column were stored at −70°C and thawed just prior to microinjection. The protein concentration of the active injected fraction was about 20 mg/ml in a buffer of 120 mM KCl, 20 mM Hepes, 1 mM EDTA, pH 7.5. Microinjection was performed on zona-intact unfertilized mouse oocyte using broken tipped micropipettes. The injection volume was 5-10 pl, estimated from the oocyte volume which is taken as 200 pl (Swann, 1990).

Measurement of intracellular calcium

Cytosolic Ca^{2+} levels were recorded using the Ca^{2+}-sensitive dyes indo-1 (Sigma) and fluo-3 (Sigma). For loading of the dyes, oocytes were incubated for 20-30 minutes at 37°C with 50 μM of the acetoxymethyl ester form of one of the two dyes in M2 medium containing 0.02% pluronic F127. After loading, oocytes were manipulated for nuclear transfer and cultured in M16 medium until the donor nucleus had fused with the oocyte. To record fluorescence, a single oocyte was placed in a 25 μl drop of M2 medium under paraffin oil in a heated chamber (32-34°C). Fluorescence was monitored with photomultiplier tubes and signals were recorded on computer using the UMANS system (Carroll and Swann, 1992). Indo-1 records are presented as the ratio of the 405 and 490 nm emission wavelength while fluo-3 signals are presented with an arbitary fluorescence scale.

Visualization of nuclei and microtubules

The oocytes were mounted on a polylysine-coated glass slide after fixation and treatment with Tween-20 (Schatten et al., 1985). For detection of tubulin, the oocytes were incubated with mouse anti-chicken α-tubulin antibody (Amersham, Bucks, UK) followed by an FITC-conjugated goat anti-mouse IgG (TAGO Inc., Burlingame, CA, USA). The chromatin was stained by incubation for 10 minutes in the DNA-specific dye, Hoechst 33258 (10 μg/ml) (Calbiochem-Behring) and observed using a Nikon Diaphot with epi-fluorescence.

RESULTS

Oocyte activation after nuclear transfer

Nuclei from fertilized 1-cell embryos were transferred to unfertilized oocytes using Sendai virus. The high levels of MPF in the intact unfertilized oocyte caused rapid NEBD and chromosome condensation of the transferred nucleus and the chromosomes became organized on a second microtubular spindle. After further culture, both sets of chromatin had resumed meiosis as shown by the formation of two separate polar bodies (2-3 hours after fusion) and two pronuclei (5-6 hours after fusion)(Fig. 1A,B). This suggested that the transplanted nucleus stimulated oocyte activation.

To investigate this phenomenon further, we transferred nuclei and cytoplasts from fertilized and parthenogenetic embryos at different stage of development (Table 1). The ability to initiate oocyte activation was specific to nuclei from fertilized 1- and 2-cell embryos. Oocyte activation was not seen after the transfer of cytoplasts from any source or after the transfer of one or two nuclei from embryos at the 4-cell stage or from cells of the inner cell mass (ICM) (Table 1). The ability to trigger oocyte activation was a property of both male and female pronuclei from fertilized 1-cell embryos. However, male pronuclei were more active since haploid nuclei from 2-cell-stage androgenetic embryos activated 86% of the recipient

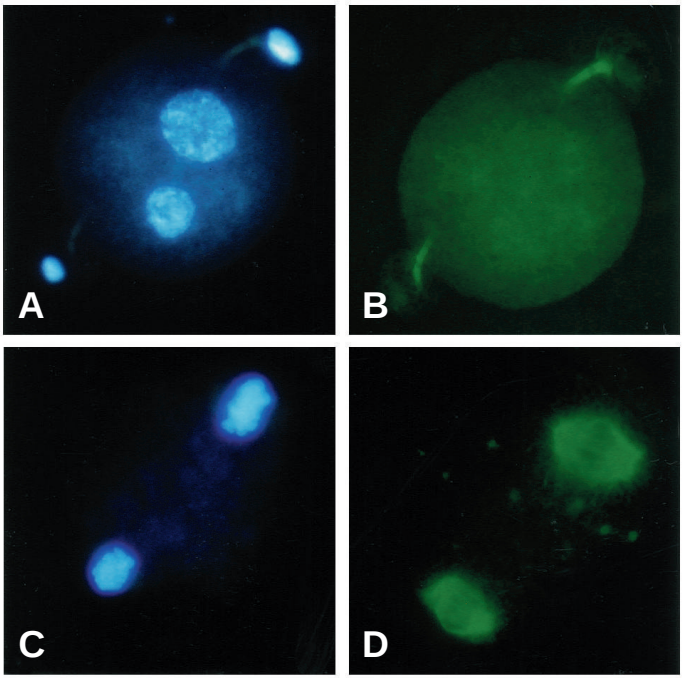

Fig. 1. The effect of transferred nuclei from fertilized and parthenogenetic embryos on chromosome and microtubule organization of unfertilized oocytes. (A) An oocyte, 6 hours after transfer of a nucleus from a fertilized 2-cell stage embryo. Note the presence of two polar bodies and two nuclei indicating oocyte activation has occurred. The larger of the two nuclei is the diploid donor, while the smaller is the haploid oocyte nucleus. (B) Distribution of tubulin in the same oocyte. The transferred nucleus underwent NEBD and premature chromosome condensation between 10 and 30 minutes after fusion with the unfertilized oocyte. Between 2 and 3 hours after fusion the second polar bodies were formed and pronuclei were visible after 6 hours. (C) An oocyte, 6 hours after transfer of a nucleus from a ethanol activated diploid parthenogenetic 2-cell embryo. Note the absence of polar bodies and nuclei, indicating oocyte activation has not been stimulated. (D) The same oocyte with two spindles. The scale bar in panel A is equivalent to 20 μm. All photomicrographs were taken at the same magnification.

Table 1. The ability of nuclei and cytoplasts from various sources to initiate activation and Ca^{2+} transients after transfer to unfertilized oocytes

Source of nucleus or cytoplast	No. oocytes activated/ Total examined	No. oocytes showing Ca^{2+} transients/ Total examined
Fertilized embryos		
Male pronuclei	41/41	4/4
Female pronuclei	41/41	4/4
2-cell nuclei	126/141	5/6
4-cell nuclei	0/36 (0/12)*	0/5
ICM cells	0/34	–
1-cell cytoplasts	0/31	–
2-cell cytoplasts	0/36	–
Cycloheximide-treated embryos		
Pronuclei	24/24	4/4
Cytoplasts	0/12	–
Androgenetic embryos		
2-cell nuclei (haploid)	18/21	–
2-cell nuclei (diploid)	14/14	–
Gynogenetic embryos		
2-cell nuclei (haploid)	13/34	–
Parthenogenetic embryos		
Ethanol activated		
Pronuclei	0/58	0/10
2-cell nuclei (diploid)	0/102	–
Cytoplasts	0/14	–
Strontium activated		
Pronuclei	0/30	0/4
Cytoplasts	0/8	–
Cytosolic sperm extract activated		
Pronuclei	12/12	8/9
Cytoplasts	6/14	–

*The number of oocytes receiving 2 nuclei from 4-cell embryos in parenthesis.

oocytes compared to haploid nuclei from gynogenetic embryos which activated only 38% of oocytes ($\chi^2_{[1]}=10.05$; $P<0.01$).

While nuclei from fertilized 1- and 2-cell embryos consistently caused oocyte activation, nuclei from similar stage parthenogenetic embryos failed to do so irrespective of whether they originated from oocyte activated by ethanol, which causes a monotonic Ca^{2+} increase (Cuthbertson et al., 1981), or strontium, which causes a series of Ca^{2+} oscillations similar to fertilization (Kline and Kline, 1992) (Table 1, Fig. 1C,D). This shows clearly that the ability of nuclei to activate the oocyte is unique to nuclei from fertilized embryos suggesting that the sperm confers Ca^{2+}-releasing activity on the nuclei of early embryos.

The role of intracellular Ca^{2+} transients in oocyte activation after nuclear transfer

Since oocyte activation is normally caused by an increase in Ca^{2+}, we investigated the possibility that nucleus-induced activation occurred by a similar mechanism. Chelation of intracellular Ca^{2+} with the specific Ca^{2+}-chelator, BAPTA, immediately after nuclear transfer, inhibited nucleus-induced oocyte activation in all cases (n=23), thereby implicating Ca^{2+} transients in nucleus-induced oocyte activation.

To investigate directly the role of changes in $[Ca^{2+}]_i$ in oocyte activation, we used the calcium-sensitive fluorescent dyes indo-1 and fluo-3 to monitor Ca^{2+} after nuclear transfer. Ca^{2+} transients were seen in all oocytes that received nuclei from fertilized 1- and 2-cell embryos (Table 1; Fig. 2A,B). The first Ca^{2+} transient occurred 40 to 70 minutes after NEBD. This was frequently followed by one to three Ca^{2+} transients at an interval of 60 to 80 minutes. Although we have not calibrated the indo-1 fluorescence recordings the Ca^{2+} transients seen after nuclear transfer were similar in magnitude and shape to Ca^{2+} transients seen at fertilization (data not shown). Consistent with the oocyte activation data, no Ca^{2+} transients were seen after the transfer of nuclei from parthenogenetic embryos (Fig. 2C), cytoplasts from any source, or after treatment of recipient oocytes with BAPTA (Fig. 2D). These experiments suggest that the Ca^{2+}-releasing activity is associated with the nucleus of fertilized embryos. To examine whether the small amount of cytoplasm transferred with the nucleus was capable of stimulating Ca^{2+} transients, pronuclei from fertilized embryos were transferred into similar stage one-cell embryos. In this case, the recipient embryos contain low amounts of MPF and the donor nucleus does not undergo NEBD. In the absence of NEBD, no Ca^{2+}

transients were seen after nuclear transfer (n=8), suggesting that NEBD is necessary for the generation of Ca^{2+} oscillations.

Pronuclei from oocytes activated by cytosolic sperm extract have Ca^{2+}-releasing activity

The finding that oocyte activation and Ca^{2+}-releasing activity is specific to nuclei from fertilized embryos suggests that the activity is a paternal contribution from the fertilizing sperm. One possible origin of the activity is a Ca^{2+}-releasing factor that has been described previously in sperm cytosol (Swann, 1990, 1994). To investigate whether the Ca^{2+}-releasing sperm factor is similar to the nucleus derived Ca^{2+}-releasing activity, pronuclei from oocytes activated by microinjection of semi-purified sperm extracts (Swann, 1994) were transferred to unfertilized oocytes. We found that pronuclei from oocytes activated with the sperm factor triggered Ca^{2+} transients and oocyte activation similar to transfer of pronuclei from fertilized embryos (Table 1, Fig. 3). We also observed that some of the cytoplasts from sperm-factor-injected oocytes caused oocyte activation. This effect was never seen after the transfer of cytoplasts from fertilized embryos suggesting that excess sperm factor was retained in a potentially active form in the cytoplasm of some of the microinjected oocytes.

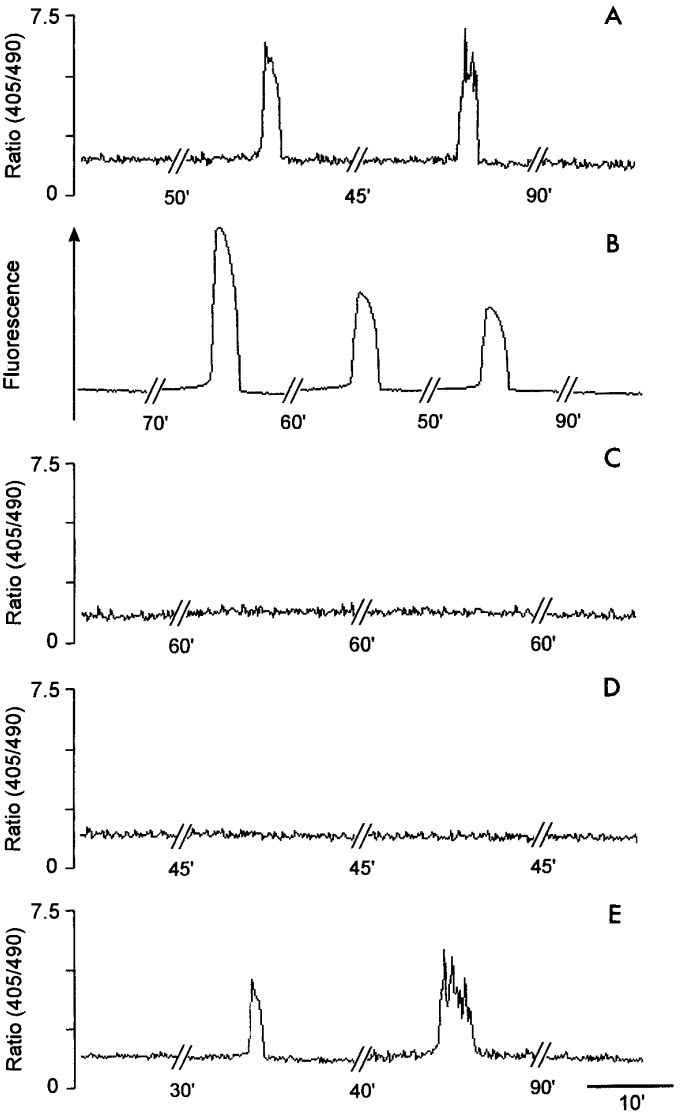

Fig. 2. Ca^{2+} transients in oocytes after the transfer of pronuclei from fertilized or parthenogenetic 1-cell embryos. The records were started 5 minutes after fusion of the oocyte and nucleus. (A,B) Measurement of intracellular Ca^{2+} transients in an oocyte after the transfer of a nucleus from a fertilized 1-cell embryo. Similar transients are seen in oocytes after the transfer of nuclei from fertilized 2-cell embryos (see Table 1). (C) Records of cytoplasmic Ca^{2+} levels in oocytes that received a pronucleus from a parthenogenetic 1-cell embryo. Note the absence of any Ca^{2+} transients. (D) The inhibition of Ca^{2+} transients after the transfer of a pronucleus from a fertilized 1-cell embryo by incubation in BAPTA/AM immediately after fusion. (E) Measurement of Ca^{2+} transients in an oocyte after transfer of a pronucleus from fertilized 1-cell embryo where protein synthesis had been inhibited continuously. Where the record has been broken, the minutes left out are indicated at each breakpoint.

The ability of nuclei to stimulate Ca^{2+} oscillations is not affected by inhibition of protein synthesis

The above results suggest that the Ca^{2+}-releasing activity may be similar to the previously described sperm factor; however, it is also possible that the sperm specifically modifies protein synthesis in the oocyte to produce an oocyte-derived calcium-releasing agent. To investigate this possibility, protein synthesis was inhibited by incubating oocytes in cycloheximide throughout fertilization, pronuclear formation and embryo manipulation. This treatment was sufficient to inhibit the incorporation of [^{3}H]leucine into protein by over 90% (Siracusa et al., 1978; Bos-Mikich et al., 1995). However, there was no effect on the ability of nuclei from cycloheximide-treated embryos to activate and trigger Ca^{2+} oscillations in recipient oocytes that had also been treated with cycloheximide (Table 1, Fig. 2E). These data suggest that oocyte activation and Ca^{2+} oscillations seen after nuclear transfer do not depend upon sperm-induced specific protein synthesis in the oocyte.

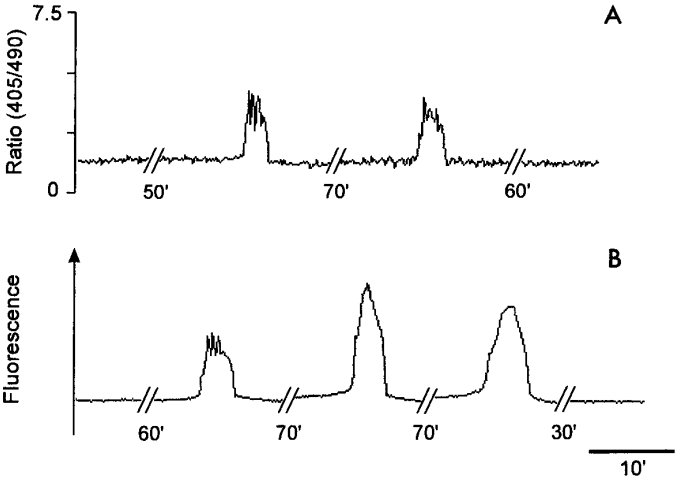

Fig. 3. Ca^{2+} transients in oocytes after the transfer of a pronucleus from a 1-cell embryo activated by the injection of a cytosolic sperm extract. (A,B) Two records from two different oocytes showing Ca^{2+} transients after the transfer of nuclei from embryos activated by injection of sperm extract.

DISCUSSION

The results of the present study show that nuclei from fertilized 1- and 2-cell embryos have Ca^{2+}-releasing activity that is sufficient to cause parthenogenetic activation after nuclear transfer. In contrast, nuclei from oocytes activated with ethanol or strontium, or cytoplasts from fertilized or parthenogenetic embryos do not stimulate Ca^{2+} oscillations or oocyte activation. However, nuclei from oocytes activated by microinjection of a Ca^{2+}-releasing cytosolic sperm extract trigger both Ca^{2+} transients and oocyte activation. These data suggest that the nuclei of fertilized 1- and 2-cell-stage embryos have a Ca^{2+}-releasing activity which is provided by the sperm at fertilization.

The origin of the Ca^{2+}-releasing activity

Our results suggest that the Ca^{2+}-releasing activity is associated with the nucleus since the transfer of similar volumes of cytoplasm fail to trigger Ca^{2+} oscillations or oocyte activation. Also a degree of nuclear reorganization appeared to be necessary for the generation of the Ca^{2+} transients since the first transient always occurred after NEBD and chromatin condensation. We cannot rule out completely the possibility that the Ca^{2+} transients seen after nuclear transfer were triggered by the associated cytoplasm rather than the components of the nucleus itself. However, we consider that this is unlikely since, in the absence of NEBD, no Ca^{2+} transients were generated and there appeared to be no obvious correlation between the Ca^{2+}-releasing activity and the size of the karyoplast transferred.

The Ca^{2+} oscillations seen after nuclear transfer have a lower frequency, but are of similar amplitude and duration to Ca^{2+} transients seen at fertilization or after sperm factor injection (Swann and Ozil, 1994; unpublished observations). A wide range of frequencies have been reported for Ca^{2+} oscillations at fertilization (Kline and Kline, 1992; Cuthbertson and Cobbold, 1985) and this variation is also seen when oocytes are fertilized in exactly the same conditions (Swann and Ozil, 1994; unpublished observations). In contrast, Ca^{2+} oscillations generated in oocytes in response to G-protein stimulation or low doses of $InsP_3$ do not show variations in frequency but are smaller in amplitude and duration (Swann, 1992, 1994). This pattern of Ca^{2+} oscillations was never observed after nuclear transfer suggesting that Ca^{2+} transients at NEBD and at fertilization are generated by a similar mechanism.

The specificity of the nuclear associated Ca^{2+}-releasing activity to nuclei from fertilized but not parthenogenetically activated oocytes suggests that the activity is of paternal origin. There are a number of possible mechanisms that might explain how the sperm confers Ca^{2+}-releasing activity upon the nucleus. Firstly, the oscillatory pattern of Ca^{2+} transients generated by the sperm at fertilization may be a necessary requirement for the Ca^{2+}-releasing activity to develop in the nucleus. Although we cannot rule out this possibility, we think it is unlikely. Ethanol triggers only a monotonic Ca^{2+} increase, but strontium-containing medium stimulates a series of repetitive transients similar to fertilization (Kline and Kline, 1992; Bos-Mikich et al., 1995). Strontium-containing medium is the most efficient means of stimulating the activation and subsequent development of freshly ovulated mouse oocytes (Bos-Mikich et al., 1995) suggesting that the activating transients also mimic the function of those seen at fertilization. Therefore, it seems unlikely that the difference in the Ca^{2+}-releasing activity between nuclei from fertilized and artificially activated embryos can be explained simply by the pattern of Ca^{2+} transients seen at fertilization.

A second possibility is that the sperm causes a factor or activity in the oocyte to associate with the developing nuclei which at NEBD is activated and stimulates Ca^{2+} release. If this is the case, it is unlikely to involve a sperm-induced effect on protein synthesis since inhibition of protein synthesis throughout fertilization had no effect on the Ca^{2+}-releasing activity of nuclei from fertilized embryos. Alternatively, the sperm might introduce an activity (e.g. a kinase) which modifies a maternal substrate that has Ca^{2+}-releasing activity and then becomes associated with the nucleus. In this case, it would be necessary for the proposed kinase to co-purify with the Ca^{2+}-releasing fraction of sperm cytosol since nuclei from oocytes activated by a microinjection of this extract also have Ca^{2+}-releasing activity.

A third possible mechanism is that the sperm directly introduces a Ca^{2+}-releasing factor at fertilization and that this factor becomes associated with the developing nuclei. This idea is supported by the presence of a Ca^{2+}-releasing activity in extracts of sperm cytosol (Swann, 1990, 1994). On microinjection into oocytes, these extracts mimic Ca^{2+} oscillations seen at fertilization. The Ca^{2+}-releasing factor appears to be a protein since it is trypsin and heat sensitive, binds to various affinity columns and has a relative molecular mass of greater than 30×10^3 (M_r) (Swann, 1990, 1994). Thus one model for explaining the ability of nuclei from early mammalian embryos to stimulate Ca^{2+} release and oocyte activation is that a protein from sperm, either directly, or through a maternal intermediary, causes the release of intracellular Ca^{2+} and then associates with the nucleus. After nuclear transfer and NEBD the active factor is released and stimulates Ca^{2+} transients and oocyte activation. Further work is required to obtain direct evidence, other than activity profiles, that the Ca^{2+}-releasing activity is localized in the nucleus. However, a number of other proteins are known to be localized to the nucleus during interphase and become cytoplasmic after NEBD. These include the cell-cycle control proteins $p55^{CDC25}$ (Millar et al., 1991), $p34^{cdc2}$-cyclin B (Ookata et al., 1992) and $p13^{suc1}$ (Hepler et al., 1994).

Another prediction of this model is that Ca^{2+} transients would be generated at NEBD of the first mitotic cell cycle of fertilized mouse embryos. Such NEBD Ca^{2+} transients have been reported recently (Tombes et al., 1992) and we have made similar observations (T. K. and J. C., unpublished data). Ca^{2+} transients are also seen during mitosis in the early embryonic cell cycles of mouse (Tombes et al., 1992), *Xenopus* (Kubota et al., 1993) and sea urchin embryos (Poenie et al., 1985; Whitaker and Patel, 1990) as well as in fibroblasts and other somatic cells (Kao et al., 1990). Furthermore, in the sea-urchin embryo, there is evidence that the NEBD Ca^{2+} transient is influenced by sperm. In polyspermic sea-urchin embryos, the Ca^{2+} transient at NEBD is larger than that seen in monospermic embryos (Whitaker and Patel, 1990). Also, cell fusion experiments in sea urchins suggest that a Ca^{2+}-releasing factor is generated after NEBD in the fertilized egg (Bennett and Mazia, 1981). It is possible that a paternally derived factor from sperm plays a role in the generation of embryonic cell cycle related Ca^{2+} transients in evolutionary divergent species.

We thank Professor T. Nakahara for support, Y. Obata and A. Bos-Mikich for technical assistance, and Dr. K. Jones for helpful discussions. This work was supported by the Medical Research Council, UK and grants-in-aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

Bennett, J. and Mazia, D. (1981). Fusion of fertilized and unfertilized sea urchin eggs. *Exp. Cell Res.* **134,** 494-498.

Bos-Mikich, A., Swann, K. and Whittingham, D. G. (1995). Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol. Reprod. Dev*. (in press).

Carroll, J. and Swann, K. (1992). Spontaneous cytosolic calcium oscillations driven by inositol trisphosphate occur during in vitro maturation of mouse oocytes. *J. Biol. Chem*. **267,** 11196-111201.

Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y. and Kohmoto, K. (1991). Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development* **113,** 789-795.

Cuthbertson, K. S. R. (1983). Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzylalcohol. *J. Exp. Zool*. **226,** 311-314.

Cuthbertson, K. S. R. and Cobbold, P. H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. *Nature* **316,** 541-542.

Cuthbertson, K. S. R., Whittingham, D. G. and Cobbold, P. H. (1981). Free Ca^{2+} increase in exponential phase during mouse oocyte activation. *Nature* **294,** 754-757.

Czolowska, R., Modlinski, J. A. and Tarkowski, A. K. (1984). Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. *J. Cell Sci.* **69,** 19-34.

Dale, B., De Felice, L. J. and Ehrenstein, G. (1985). Injection of a soluble sperm extract into sea urchin eggs triggers the cortical reaction. *Experimentia* **41,** 1068-1070.

Fulton, B. P. and Whittingham, D. G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature* **273,** 149-151.

Hashimoto, N. and Kishimoto, T. (1988). Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Dev. Biol*. **126,** 242-252.

Hepler, P. K., Sek, F. J. and John, P. C. L. (1994). Nuclear localization and mitotic dispersion of the essential cell cycle protein $p13^{suc1}$ examined in living cells. *Proc. Natl. Acad. Sci. USA* **91,** 2176-2180.

Jaffe, L. A. (1983). Sources of calcium in egg activation; a review and hypothesis. *Dev. Biol*. **99,** 265-276.

Jaffe, L. (1990). First messengers at fertilization. *J. Reprod. Fert.* **42 Supplement,** 107-116.

Kao, J. P. Y., Alderton, J. M., Tsien, R. Y. and Steinhardt, R. A. (1990). Active involvement of Ca^{2+} in mitotic progression of swiss 3T3 fibroblasts. *J. Cell Biol.* **111,** 183-196.

Kline, D. and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149,** 80-89.

Kono, T., Kwon, O. Y. and Nakahara, T. (1991). Development of enucleated mouse oocytes reconstituted with embryonic nuclei. *J. Reprod. Fert.* **93,** 165-172.

Kono, T., Sotomaru, Y., Sato, Y. and Nakahara, T. (1993). Development of androgenetic mouse embryos produced by in vitro fertilization of enucleated oocytes. *Mol. Reprod. Dev.* **34,** 43-46.

Kubota, H. Y., Yoshimoto, Y. and Hiramoto, Y. (1993). Oscillation of intracellular free calcium in cleaving and cleavage-arrested embryos of *Xenopus laevis. Dev. Biol*. **160,** 512-518.

Lorca, T., Cruzalegui, F. H., Fesquest, D., Cavadore, J-C., Mery, J., Means, A. and Doree, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366,** 270-273.

Masui, Y. (1991). The role of cytostatic factor (CSF) in the control of oocyte cell cycle: A summary of 20 years of study. *Develop. Growth Differ*. **33,** 543-551.

Millar, J. B. A., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C. and Russell, P. (1991). $p55^{CDC25}$ is a nuclear protein required for the initiation of mitosis in human cells. *Proc. Natl. Acad. Sci. USA* **88**, 10500-10504.

Miyazaki, S-I., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. (1986). Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev. Biol*. **118,** 259-267.

Miyazaki, S-I., Shirakawa, H., Nakade, K. and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor? Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev. Biol.* **58,** 62-78.

Ookata, K., Hisanaga, S., Okano, T., Tachibana, K. and Kishimoto, T. (1992). Relocalization and distinct subcellular localization of $p34^{cdc2}$-cyclin B complex at meiosis reinitiation in starfish oocytes. *EMBO J.* **11,** 1763-1772.

Ozil, J. P. (1990). The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* **109,** 117-127.

Poenie, M., Alderton, J. Tsien, R. Y. and Steinhardt, R. A. (1985). Changes in free calcium with stage of the cell division cycle. *Nature* **315,** 147-149.

Sagata, N., Watanabe, N., Woude, G. F. V. and Ikawa, Y. (1989). The *c-mos* proto-oncogene is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342,** 512-518.

Schatten, G., Simerly, C. and Schatten, H. (1985). Microtubule configuration during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. *Proc. Natl. Acad. Sci. USA* **82,** 4152-4156.

Siracusa, G. Whittingham, D. G., Molinaro, M. and Vivarelli, E. (1978). Parthenogenetic activation of mouse oocytes induced by inhibitors of protein synthesis. *J. Embryol. Exp. Morph.* **43,** 151-166.

Stice, S. L. and Robl, J. M. (1990). Activation of mammalian oocyte by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.* **25,** 272-280.

Surani, M. A. H., Barton, S. C. and Norris, M. L. (1986). Nuclear transplantation in the mouse: Heritable differences between parental genomes after activation of the embryonic genome. *Cell* **45,** 127-136 (1986).

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110,** 1295-1302.

Swann, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine sensitive store. *Biochem. J.* **287**, 79-84.

Swann, K. (1994). Ca^{2+} oscillations and sensitization of Ca^{2+} release in unfertilized mouse eggs injected with a sperm factor. *Cell Calcium* **15,** 331-339.

Swann, K. and Ozil, J. P. (1994). Dynamics of the calcium signal that triggers mammalian egg activation. *Int. Rev. Cytol.* **152,** 183-222.

Taylor, C. T., Lawrence, M. Y., Kingsland, C. R., Biljan, M. M. and Cuthbertson, K. S. R. (1993). Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. *Human Reprod.* **8,** 2174-2179.

Tombes, R. M., Simerly, C., Borisy, G. C. and Shatten, G. (1992). Meiosis, egg activation, and nuclear envelope breakdown are differentially reliant on Ca^{2+}, whereas germinal vesicle breakdown is Ca^{2+} independent in the mouse oocyte. *J. Cell Biol.* **117,** 799-811.

Toyoda, Y., Yokoyama, M. and Hosi, T. (1971). Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. *Jpn. J. Anim. Reprod.* **16,** 149-151.

Tsunoda, Y., Tokunaga, T., Imai, H. and Uchida, T. (1989). Nuclear transplantation of male primordial germ cells in the mouse. *Development* **107,** 407-411.

Vincent, C., Cheek, T. R. and Johnson, M. H. (1992). Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *J. Cell Sci.* **103,** 389-396.

Whitaker, M. and Patel, R. (1990). Calcium and cell cycle control. *Development* **108,** 525-542.

Whittingham, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert.* **14 Supplement,** 7-21.

Whittingham, D. G. (1980). Parthenogenesis in mammals. In *Oxford Reviews of Reproductive Biology.* pp 205-231. Oxford Univ. Press.

(Accepted 7 December 1994)