Journal of Cell Science 108, 143-151 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The exit of mouse oocytes from meiotic M-phase requires an intact spindle during intracellular calcium release

Nicola J. Winston[1,*], Orla McGuinness[2], Martin H. Johnson[3] and Bernard Maro[1]

[1]Departement de Biologie du Développement, Institut Jacques Monod, CNRS-Université Paris VII, Tour 43, 2 Place Jussieu, 75005 Paris, France
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
[3]Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK

*Author for correspondence

SUMMARY

To study the role of the metaphase spindle during the period of oocyte activation, mouse oocytes were fertilised or activated parthenogenetically in the presence or absence of the microtubule inhibitor nocodazole. In both cases, nocodazole caused the disappearance of the spindle and prevented the passage of the oocytes into interphase. However, the calcium spiking responses of the oocytes were not affected by nocodazole, being repetitive after fertilisation and a single spike after activation. If, after their activation or fertilisation in nocodazole, oocytes were later removed from the drug, only those that had been fertilised progressed into interphase. This progress was associated with continuing calcium spiking. Moreover, both the spiking and the progress to interphase could be blocked or reduced in incidence by removal of external calcium or addition of 5,5′-dimethyl BAPTA-AM. Oocytes that had been activated by ethanol in the presence of nocodazole and then removed from it, to allow re-formation of the spindle, only progressed into interphase if given a second exposure to ethanol, thereby eliciting a second calcium transient. These results show that exit from meiotic M-phase requires the simultaneous presence of a fully intact spindle during the release of calcium and that those factors leading to the degradation of cyclin B are only activated transiently. Since cyclin is being degraded continuously in the metaphase-II-arrested mouse oocyte and since this degradation is microtubule-dependent, these data suggest that the superimposition of a high concentration of intracellular calcium is required to tilt the equilibrium further in favour of cyclin degradation if exit from M-phase is to occur.

Key words: mouse, oocyte, spindle, microtubule, activation, calcium

INTRODUCTION

The mature ovulated murine oocyte is arrested in metaphase of the second meiotic division unless activated by fertilisation, which triggers a series of transient rises in intracellular calcium concentration (Cuthbertson and Cobbold, 1985; Swann, 1990; Cheek et al., 1993). These calcium transients drive resumption of the cell cycle by inactivating M-phase promoting factor (MPF) (Newport and Kirschner, 1984; Murray et al., 1989; Watanabe et al., 1991; Kline and Kline, 1992). Cyclin is the regulatory subunit of MPF and its destruction is required for exit from M-phase (Nurse and Bissett, 1981; Gerhart et al., 1984; Lohka et al., 1988; Draetta et al., 1989; Labbe et al., 1989). In mouse oocytes, during the metaphase II arrest, cyclin B turns over and its destruction requires the presence of an intact spindle, since exposure to the microtubule-depolymerising drug nocodazole is associated with a reduced rate of cyclin B degradation (Kubiak et al., 1993). It has been proposed, therefore, that cyclin B and the enzyme(s) that degrade it must be brought together physically on the spindle for effective degradation and entry into interphase to occur (Kubiak et al., 1993).

In fertilised mouse oocytes, the entry into interphase can be delayed if fertilisation occurs in the presence of nocodazole, which holds the oocyte in a protracted metaphase arrest. During this period of nocodazole-induced arrest, the chromosomes are freed from the dismantled spindle and disperse around the cortex of the oocyte by a mechanism that is dependent on an intact system of microfilaments. If, subsequently, such oocytes are removed from nocodazole, microtubules assemble around each group of chromosomes to form a series of small metaphase-like spindles located near the cortex. Each spindle then completes the meiotic division, several polar bodies are extruded and the oocyte passes into interphase (Maro et al., 1986). Thus, during the period of nocodazole arrest, the oocyte has retained the ability to respond to the fertilisation stimulus until conditions suitable for development prevail. Oocytes may also be activated parthenogenetically by a variety of stimuli, most of which seem to induce a calcium spike (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992; Vincent et al., 1992; Vitullo and Ozil, 1992; Cheek et al., 1993; Gallicano et al., 1993; Shiina et al., 1993; Colonna et al., 1989) and thereby activate exit from M-phase by a mechanism similar to that stimulated by spermatozoa. In

this paper we show that the presence of nocodazole also prevents exit from M-phase during parthenogenetic activation, but that, in contrast, removal of the drug subsequently is not followed by entry of oocytes into interphase. This difference between parthenogenetic and sperm stimuli is explained in terms of the different patterns of calcium spiking induced. We propose that the calcium pulse must occur in the presence of an intact spindle for exit from M-phase.

MATERIALS AND METHODS

Recovery and handling of oocytes used for immunocytochemical analysis

Six- to 10-week-old female Swiss mice (Animalerie Spécialisée de Villejuif, Centre Nationale de la Recherche Scientifique, France) were superovulated by intraperitoneal injections of 5 i.u. of pregnant mare's serum gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet), 48 hours apart.

Oocytes to be activated with ethanol were recovered by puncturing the ampullae of oviducts at 13 hours post-hCG. The cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma), zonae pellucidae removed with acid Tyrode's solution (Nicholson et al., 1975) and the oocytes were cultured in M2 + BSA (Fulton and Whittingham, 1978) under paraffin oil at 37°C for 4-5 hours. At 18 hours post-hCG, some oocytes were then exposed directly to 8% ethanol (Merck) with or without nocodazole (Sigma; stock solution 10 mM in DMSO stored at –20°C) for 6 minutes, rinsed three times and placed into drops of M2 + BSA under paraffin oil for a further 4 to 6 hours. Other oocytes were incubated in nocodazole for 15, 30, or 60 minutes prior to ethanol treatment at 18 hours post-hCG as described above.

For fertilisation in vitro, either cumulus-enclosed or zona-free oocytes were used. Cumulus masses were released 12.5 hours post-hCG, directly into insemination drops of Toyoda's medium (Toyoda et al., 1971), containing 10% foetal calf serum (FCS) with or without 10 μM nocodazole under paraffin oil equilibrated overnight in 5% CO_2 at 37°C. The cumulus masses were incubated for at least 1 hour before addition of 90-120 ml of spermatozoa (to a final concentration of $1\text{-}2\times10^6$ motile sperm/drop) at approximately 13.5 hours post-hCG. Zona-free oocytes were obtained as described above and inseminated in 1 ml drops of M2 + BSA with or without nocodazole under oil, using either 1×10^6 or 1×10^5 motile sperm/drop. Spermatozoa were recovered 2 hours prior to their use from the cauda-epididymides of CBA mice into 0.5 ml drops of Toyoda's medium + 10% FCS, and were incubated at 37°C for 1.5 hours to capacitate (Fraser and Drury, 1975; Fraser, 1983).

Approximately 3 hours post-insemination (hpi), control oocytes not exposed to nocodazole were transferred to sperm-free culture medium M2 + BSA containing 1.78 mM Ca^{2+}. Oocytes inseminated in nocodazole were selected at random and washed directly via three rinses into normal calcium-containing medium with or without 10 mM 5,5′-dimethyl-BAPTA-AM (Molecular Probes), which enters the cell, is hydrolysed to BAPTA and buffers elevations of intracellular calcium without depressing basal levels (Kline and Kline, 1992). Other oocytes were washed into calcium-free medium M2 + BSA, containing nocodazole. After 30 minutes, these latter oocytes were washed out of nocodazole into calcium-free medium alone. Half of these oocytes were retained in calcium-free medium, whilst the remainder were returned to calcium-containing medium after 1 hour. All oocytes were cultured for a further 6 hours after which the zonae were removed from those oocytes that had been fertilised in their cumulus mass. Development was scored immunocytochemically and defined as either: (i) activated (having one pronucleus if ethanol activated, two pronuclei if fertilised, or more than two pronuclei if fertilised in the presence of nocodazole); or (ii) non-activated (having one, or more in nocodazole-treated oocytes groups of condensed chromosomes each with a metaphase spindle). The behaviour of zona-intact and zona-free oocytes treated and cultured in vitro did not differ and thus are presented together in Results.

Oocyte fixation and immunocytochemical staining

Zona-free oocytes were placed in specially designed chambers (see Maro et al., 1984) coated with 0.1 mg/ml concanavalin A (Sigma). The samples were centrifuged at 450 ***g*** for 10 minutes at 37°C and then fixed as described by de Pennart et al. (1988) with 0.1% glutaraldehyde (Sigma) in PBS containing 1% Triton X-100 (Boehringer Mannheim GmbH). After rinsing for 5 minutes in PBS, the samples were extracted with 2% Triton X-100 for 30 minutes at room temperature, incubated for 3 periods of 10 minutes in 10 mg/ml $NaBH_4$ in PBS and processed for immunofluorescence as described by Maro et al. (1984). The tubulin was visualised using the rat monoclonal antibody YL1/2 to tyrosinated α-tubulin (Kilmartin et al., 1982), followed by a fluorescein-labelled anti-rat antibody (Miles). Chromatin was stained with propidium iodide (Molecular Probes; 5 mg/ml in PBS).

Microscopy

The coverslips were removed from the chambers and mounted on glass slides in AF1 mountant (Citifluor, London) to prevent bleaching. The samples were examined and scored for activation using a Leitz Diaplan epifluorescence microscope equipped with ×63 (Plan Apo; NA 1.4) and ×100 (Plan Fluotar; NA 1.32) objectives. Photomicrographs were taken on Ektachrome 160 film using a Bio-Rad MRC-600 confocal microscope mounted on an Optiphot II Nikon microscope equipped with a ×60 objective (Plan Apo; NA 1.4) and a dual laser system (argon and helium-neon) adjusted at excitation wavelengths of 488 and 534 nm, respectively. The signal was averaged using a Kalman filter (on 8 images).

Histone H1 kinase assay

Histone H1 kinase activity was determined in HK buffer (80 mM β-glycerophosphate, 20 mM EGTA, pH 7.3, 15 mM $MgCl_2$, 1 mM DTT, 10 mM leupeptin, 1 mM pepstatin, 10 mM aprotinin, 0.2 mM AEBSF) using exogenous histone H1 (HIII-S from calf thymus, Sigma) as substrate. Samples each containing 8 oocytes in 1 ml medium M2 + BSA were diluted fourfold in twice concentrated HK buffer, lysed by freezing and thawing twice and the reaction was started by the addition of a solution containing 3.3 mg/ml histone H1, 1 mM ATP and 0.95 mCi/ml [γ-^{32}P]ATP. Incubation continued for 30 minutes at 30°C and the reaction was stopped by adding twice concentrated sample buffer (Laemmli, 1970) and boiling for 5 minutes. The samples were then analysed by electrophoresis in 15% polyacrylamide gels containing 0.1% SDS followed by autoradiography. The autoradiograms were scanned and analysed using image analysis software (Image 1.54 by Wayne Rasband, NIH).

Measurement of intracellular calcium using fura-2

Oocytes for fura imaging were collected from MF1 female mice (OLAC) and spermatozoa were obtained from F1LAC males (C57Bl.10 females × CBA/Ca males bred in the Department of Anatomy, Cambridge). Oocytes were processed for fertilisation and activation as above except that: removal of the zona pellucida was achieved by brief exposure to α-chymotrypsin, Whittingham's medium (Whittingham, 1971) was used for sperm capacitation, and modified medium H6 + BSA (Nasr-Esfahani et al., 1990) was used for handling and washing. Oocytes were mounted in special chambers and loaded with fura-2-AM (2 mM; membrane-permeable form, Molecular Probes) for 30 minutes exactly as described by Vincent et al. (1992). For oocytes incubated in nocodazole for 1 hour before insemination, the fura-2 was added to the medium during the last half hour of incubation. All media used contained 1.78 mM

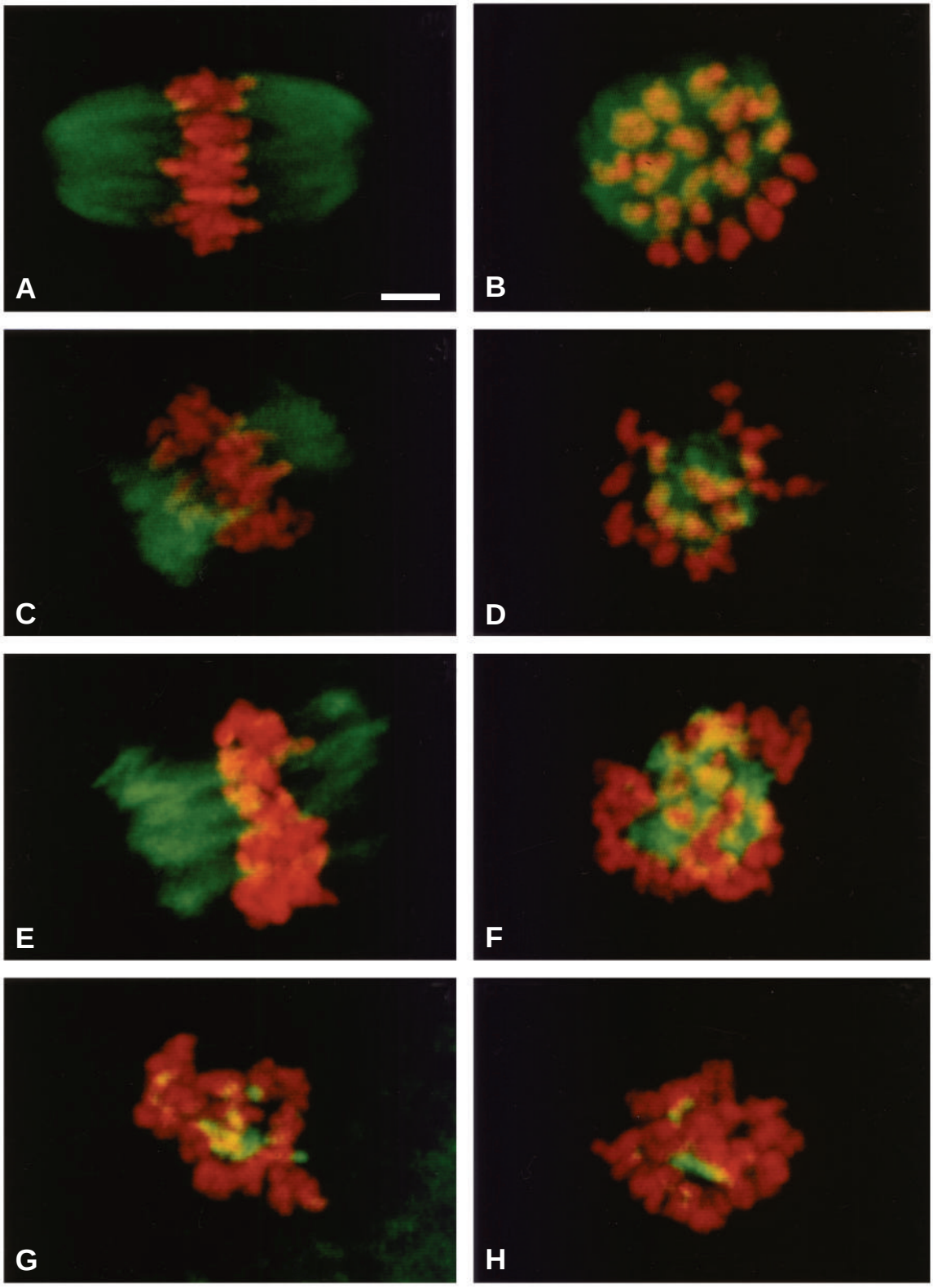

Fig. 1. Pairs of confocal images showing the kinetics of spindle disassembly in oocytes treated with nocodazole for various periods of time viewed both side-on (A,C,E and G) and en face (B,D,F and H), with the microtubules stained green and the chromosomes stained red (areas of overlap of these structures appear yellow). (A,B) Control untreated oocytes; (C,D) 1 minute; (E,F) 3 minutes; (G,H) 6 minutes of nocodazole treatment. Bar, 4 μm.

calcium. The chamber containing the oocytes was then placed in a well on the stage of a Nikon Diaphot TMD inverted epifluorescence microscope for imaging. Incubations were all carried out via a system of perfusion through the perfusion chamber maintained at 37°C.

fura-2 imaging

Intracellular free calcium activity $[Ca^{2+}]i$ was imaged through a Nikon CF-Fluor 20× objective and intensified CCD camera (Extended ISIS, Photonic Science, Robertsbridge, UK), by calculating the ratio of fura-2 fluorescence at 510 nm, excited by UV light alternately at 340 and 380 nm from twin xenon arc lamps and grating monochromators. Excitation wavelengths were alternated by a rotating chopper mirror attached to a stepped-motor, which was driven in synchrony with the video signal from the camera, to switch wavelengths at the end of each video frame. The resulting video signals were combined by an 'Imagine' digital image processor (Synoptics Ltd, Cambridge, UK) using a look-up table to implement the formula of Grynkiewicz et al. (1985). The calculation was done in real time, to give a 'live' image of $[Ca^{2+}]_i$, which was updated every 80 milliseconds, and smoothed by recursive filtering with a 200 millisecond time-constant to reduce the noise (see O'Sullivan et al., 1989; Moreton, 1991). The live image was recorded continuously on video tape, and subsequently played back and re-digitized into frame store, using software written in the semper language (Synoptics Ltd) to sample selected oocytes, and to record and plot mean $[Ca^{2+}]_i$ readings at regular time intervals. In all cases data were sampled at 5-second intervals. The precise values measured can vary slightly between individual oocytes however, the pattern of the readings over time were consistent within each treatment group. Figs 2, 5 and 6 show representative traces from a single oocyte treated as described in the legend. Oocyte morphology was scored under phase-contrast in the chambers during and after the treatment period.

RESULTS

Nocodazole prevents oocyte activation when present during ethanol stimulation

In mouse oocytes the presence of the metaphase spindle is required for cyclin destruction and thus exit from M-phase (Kubiak et al., 1993). This led us to examine the role of the spindle microtubules during the period of activation using the microtubule-depolymerising drug nocodazole. Immunocytochemical staining of oocytes after 1 to 6 minutes of treatment with nocodazole revealed that their spindles were reduced within 1 minute of exposure and only traces of stained tubulin remained after 6 minutes (n=80-100 from three experiments: Fig. 1).

Oocytes for activation were recovered 13 hours after the injection of hCG, cultured in vitro for up to 5 hours and divided into six groups: (i) control untreated oocytes; (ii) exposed to 8% ethanol alone; or (iii) ethanol and nocodazole simultaneously for 6 minutes, or treated with nocodazole for (iv) 15 minutes, (v) 30 minutes and (vi) 60 minutes prior to exposure to ethanol plus nocodazole for 6 minutes. Oocytes from all groups were then washed into normal culture medium for up to 4 hours before immunocytochemical analysis. No activation occurred in the control group or in those oocytes pre-treated with nocodazole (Table 1). The incidence of activation in oocytes exposed simultaneously to both ethanol and nocodazole was reduced significantly (P=<0.05; chi^2) compared to oocytes treated with ethanol alone.

Table 1. Incidence of activation in groups of oocytes activated parthenogenetically in the presence or absence of nocodazole or after pre-treatment with nocodazole

Activation medium (6 min)	Nocodazole pre-treatment (min)	Incidence of activation (%)	Number of experiments
(i) Control	0	0/193 (0)	7
(ii) Ethanol only	0	141/184 (71)	7
(iii) Ethanol and nocodazole	0	60/175 (36)	6
(iv) Ethanol and nocodazole	15	0/49 (0)	1
(v) Ethanol and nocodazole	30	0/66 (0)	2
(vi) Ethanol and nocodazole	60	0/65 (0)	3

A similar effect was observed when the calcium ionophore A23187 was used instead of ethanol to induce the activating stimulus; 51% of 74 oocytes were activated when treated with ionophore alone for 5 minutes, compared to 5% of 78 oocytes exposed to ionophore plus nocodazole (data not shown).

Video imaging of fura-loaded oocytes exposed to ethanol alone (n=22, 2 replicates) or after 60 minutes of exposure to nocodazole prior to ethanol treatment (n=32), revealed in both cases a large single calcium transient during the period of ethanol exposure (Fig. 2A,B).

When oocytes that had been exposed to nocodazole before being treated with ethanol in the presence of nocodazole and transferred to control medium, were then re-exposed to ethanol alone 1 hour later, a second calcium release was observed under imaging conditions (Fig. 2C); 100% of oocytes treated identically in vitro had extruded the second polar body within 2.5 hours of the second ethanol treatment (n=35 + 1 cleaved immediately to the 2-cell stage). Immunocytochemical staining confirmed that the oocytes were in telophase, with decondensing groups of chromosomes separated by a microtubular midbody.

Histone H1 kinase activity is high in control non-activated oocytes (Fig. 3, column 1), but declines rapidly after activation (Fig. 3, column 2; Kubiak et al., 1993). Incubation of oocytes in nocodazole followed by exposure to ethanol or ionophore at levels that stimulate a calcium transient did not result in a decline in H1 kinase activity (Fig. 3, columns 3-5). However, if oocytes that had been exposed transiently to ethanol in nocodazole were then rinsed free of nocodazole and re-exposed to ethanol, their H1 kinase activity was reduced (Fig. 3, column 6).

Table 2. Incidence of activation in groups of oocytes inseminated in the presence of nocodazole and then removed to medium with or without calcium or the calcium buffering agent BAPTA-AM

Treatment group	Incidence of activation (%)	Number of experiments	Mean ± s.d.
Control	185/447 (41)	6	44.6±16.5
Nocodazole, then control	105/429 (24)	6	25.0±7.6
Nocodazole, then BAPTA-AM	2/249 (1)	3	0.9±1.0
Nocodazole, then Ca (–)*	12/226 (5)	4	5.4±2.7
Nocodazole, then Ca (–)* then Ca (+)†	63/217 (29)	4	31.2±10.6

*Ca (–), calcium-free medium.
†Ca (+), calcium-containing medium.

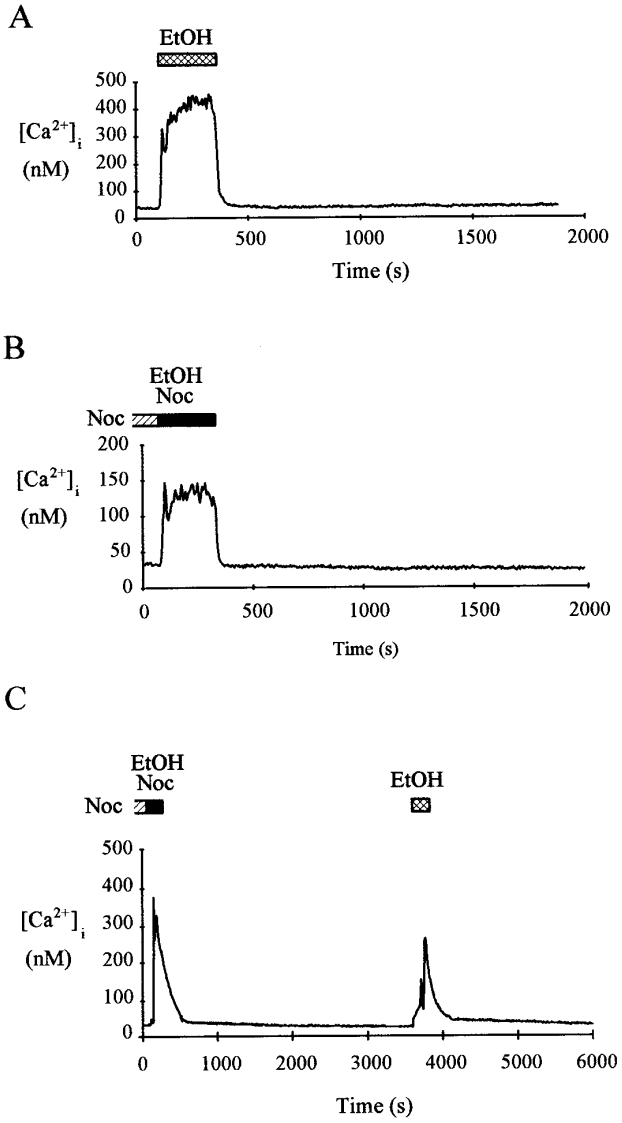

Fig. 2. (A) Oocytes exposed to ethanol released a single calcium spike and exited from M-phase, whilst oocytes preincubated in nocodazole and exposed to ethanol in the presence of nocodazole also released a single calcium spike (B), but failed to exit M-phase. (C) One hour after removal from nocodazole, a second exposure to ethanol induced a calcium transient and exit from M-phase.

Fertilisation of oocytes in nocodazole induces calcium spiking

In confirmation of our previous results (Maro et al., 1986), oocytes inseminated in the presence of nocodazole were fertilised, but they resumed meiosis only after removal from nocodazole, at which time they extruded multiple polar bodies, and formed pronuclei and an interphase network of microtubules (Table 2, line 2; Fig. 4D, compare with control fertilised oocytes, Table 2, line 1; Fig. 4C, and with unfertilised control, Fig. 4A, and nocodazole-treated, Fig. 4B, oocytes). We had observed previously that in oocytes treated with nocodazole for 2 hours and then removed from the drug miniature spindles re-formed within 60 minutes (n=30). Oocytes loaded with fura-2-AM and inseminated in medium containing nocodazole showed a calcium-spiking pattern (n=18/22) indistinguishable from that of controls. The mean amplitude of the third spike was 291±35 nM and its mean width was 54.7±3 seconds (compare with values of 318±36 nM and 50±4 seconds for controls, respectively; Cheek et al., 1993). The spiking continued after removal of the nocodazole (n=16/18; Fig. 5A,B).

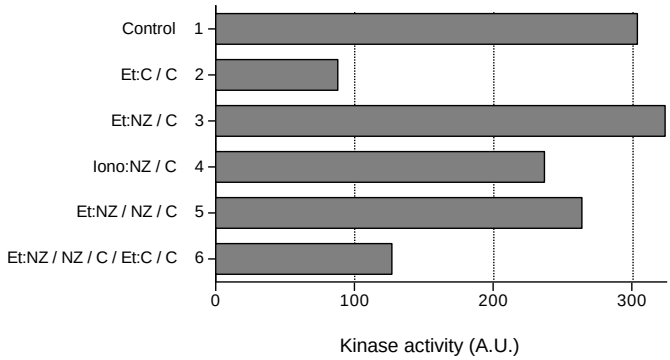

Fig. 3. Values for the activity of the histone H1 kinase in control unactivated oocytes (1), oocytes activated in ethanol (2), oocytes exposed to an ethanol (3 and 5) or ionophore (4) activation stimulus in the presence of nocodazole, and oocytes exposed first to ethanol in the presence and then in the absence of nocodazole (6). All oocytes cultured in medium M2 + BSA + nocodazole for 1 hour and then: control, collected immediately; Et:C, ethanol in M2 + BSA (6 minutes); /, followed by: C, M2 + BSA alone (1 hour); Et:NZ, ethanol in M2 + BSA + nocodazole (6 minutes); Iono:NZ, calcium ionophore A23187 in M2 + BSA + nocodazole (5 minutes); NZ, M2 + BSA + nocodazole (1 hour). A.U., arbitrary units.

Prevention of internal calcium spiking blocks oocyte activation after fertilisation

To determine whether calcium spikes were involved in the resumption of meiosis in oocytes fertilised in the presence of nocodazole and then removed from the drug, oocytes selected randomly from an insemination drop containing nocodazole were depleted of calcium in one of two ways.

(i) Some oocytes were washed out of nocodazole directly into medium containing the permeant intracellular calcium chelator BAPTA-AM (Kline and Kline, 1992). Only two of 249 oocytes in 3 experimental replicates showed signs of exit from M-phase over the following 6 hours, when examined immunocytochemically (Table 2, line 3). Parallel video imaging of 10 fura-loaded oocytes that had been fertilised in nocodazole and exhibited calcium spiking revealed that on transfer into nocodazole-free medium containing BAPTA-AM, the spiking ceased (Fig. 6A; one oocyte showed a single calcium spike) and none exited into interphase. The chromosomes in all oocytes arrested in M-phase in the presence of BAPTA-AM were dispersed in clumps and condensed. Each clump had recruited some microtubules, but had not organised them into fully formed miniature spindles, as in the case in non-activated oocytes washed out of nocodazole into normal culture medium (compare Fig. 4B and E). This result raised the possibility that failure to exit M-phase in BAPTA-AM reflected failure to form a spindle. However, when the same experiment was performed with oocytes inseminated in the presence of both nocodazole and cytochalasin D (to prevent

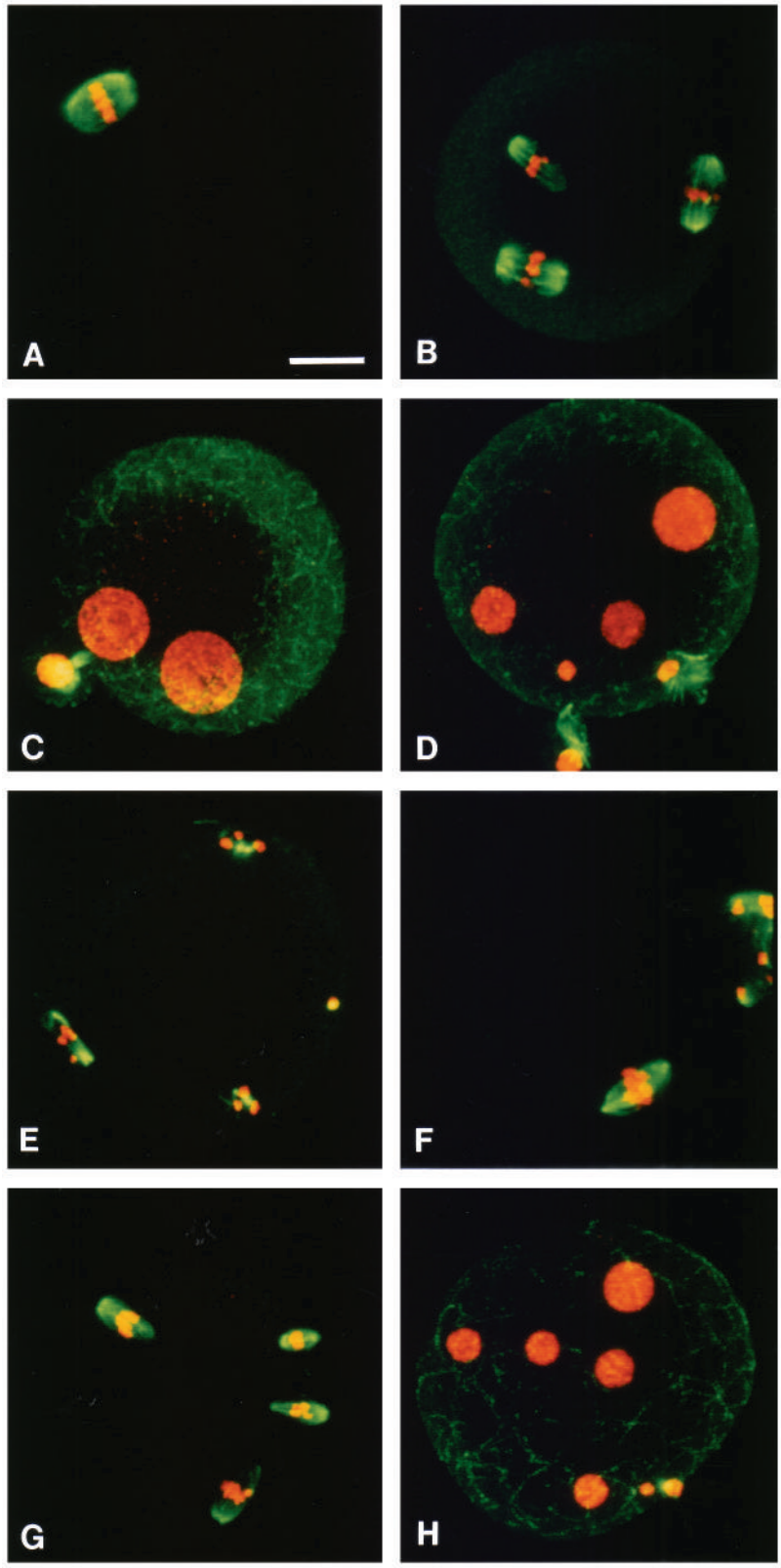

Fig. 4. The chromosomes of non-fertilised oocytes are condensed and arranged on the metaphase plate of a barrel-shaped spindle (A). In unfertilised oocytes treated with nocodazole for 3-4 hours and then washed free of nocodazole for 6 hours, the chromosomes were condensed and dispersed around the cortex of the oocyte in 2 to 6 small clumps, each of which had recruited polymerised microtubules into a miniature, but otherwise fully formed meiotic spindle (B). Fertilised control oocytes formed a single polar body (C), whilst oocytes fertilised in nocodazole, and then removed from it, extruded multiple polar bodies (D); both passed into interphase. Nocodazole-treated oocytes, on removal from the drug to a medium containing BAPTA-AM, had multiple groups of condensed chromosomes, but failed to form complete spindles (E); addition of cytochalasin D (CCD) to the nocodazole-containing fertilisation medium prevented chromosome dispersal and, on transfer to nocodazole-free medium containing BAPTA-AM, a single spindle with pointed poles was formed around the single group of chromosomes (F). Oocytes depleted of internal calcium stores in calcium-free medium containing nocodazole and then cultured in calcium-free medium alone remained mostly arrested in M-phase, and full minature spindles formed around each group of dispersed chromosomes (G). If some of these oocytes were returned to calcium-containing medium after 1 hour, they were able to exit M-phase, extrude multiple polar bodies and pass into interphase (H). Bar, 16.6 μm.

the microfilament-dependent dispersal of the chromosomes, which remain in a single clump; Maro et al., 1986), a full-sized spindle formed around the single group of condensed chromosomes. However, the spindle poles were pointed (Fig. 4F), rather than having the usual barrel shape (Fig. 4A). Nonetheless, of 186 such oocytes, washed into BAPTA-AM, only 1% exited M-phase, compared to 51/196 (26%) of controls.

(ii) In a second group of oocytes taken from insemination medium containing nocodazole, internal calcium stores were exhausted in calcium-free medium containing nocodazole,

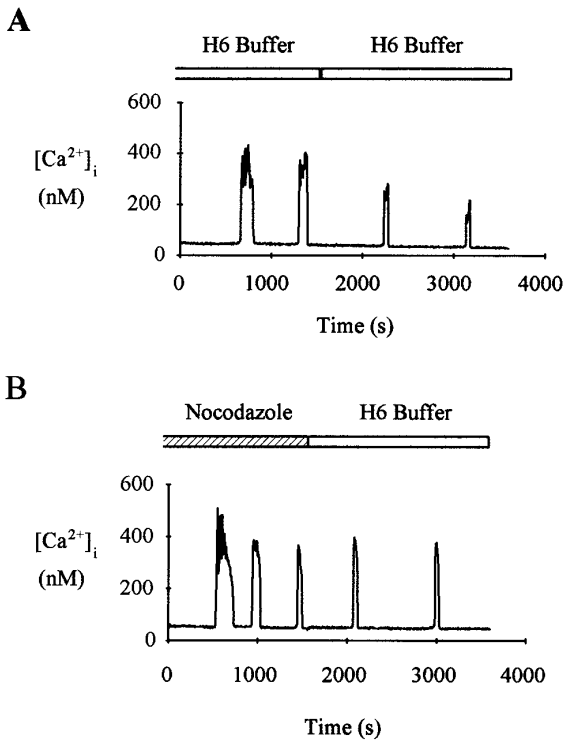

Fig. 5. (A) Fertilised control oocytes show transient rises in intracellular calcium. (B) Oocytes fertilised in the presence of nocodazole (hatched bar) also showed calcium transients indistinguishable from those seen in control oocytes and which continued after the removal of the drug (open bar).

after which they were transferred to calcium-free medium alone. After 1 hour, some of these oocytes were returned to calcium-containing medium for 5 hours and the rest were retained in calcium-free medium. Of 217 oocytes returned to calcium, 29% overall extruded a polar body and exited to interphase (Fig. 4H; Table 2, line 5, similar to the group of oocytes treated with nocodazole alone, 24%; *n*=429; Table 2, line 2) compared with 5% of those remaining in calcium-free medium (*n*=226; Table 2, line 4). All oocytes that remained arrested in M-phase had condensed chromosomes dispersed between 3 to 4 clumps, each of which had recruited polymerised microtubules into fully formed miniature spindles (Fig. 4G).

fura-imaging of 10 oocytes fertilised in nocodazole revealed calcium transients, which ceased after removal of calcium and nocodazole. Re-addition of calcium resulted in the resumption of spiking in 8 oocytes, showing either a single spike (5 oocytes; Fig 6B) or multiple spikes (3 oocytes; Fig. 6C). Polar bodies were observed in 2 oocytes that resumed calcium spiking (one with a single spike and one with multiple spikes). In 3 other oocytes, polar body structures were observed before removal of nocodazole, suggesting that they were already activated prior to nocodazole exposure.

DISCUSSION

The experiments reported here were designed to test the proposition that both an intact spindle and a pulse of calcium must coincide to allow exit of arrested mouse oocytes from M II into interphase (Kubiak et al., 1993). Our data show that oocytes incubated in nocodazole, and then fertilised or parthenogenetically activated in its presence, failed to progress to interphase. We also show that they responded with calcium transient patterns indistinguishable from those in control oocytes not exposed to nocodazole. Thus, their failure to exit M-phase is not due to a failure of the calcium-spiking mechanism in the presence of nocodazole.

We confirmed our earlier observation (Maro et al., 1986) that removal of nocodazole from fertilised oocytes allowed them to re-form spindles and progress to interphase. During this period, the calcium spiking initiated at fertilisation continued. In contrast, whilst oocytes exposed to ethanol in nocodazole did re-form intact spindles after the removal of nocodazole, they did not show further spiking and did not exit M-phase. However, a second exposure to ethanol induced a second calcium spike and entry into interphase. Analysis of the activity of the histone H1 kinase in these oocytes confirmed that the failure to exit from M-phase was always accompanied by high levels of H1 kinase activity. In contrast, those oocytes possessing an intact spindle and capable of responding normally to the activation stimulus showed a dramatic fall in the level of H1 kinase activity. It thus seemed reasonable to conclude that it was the coincidence of an intact spindle and a calcium transient that was required for the transition to interphase and that the difference in responses to nocodazole withdrawal between fertilised and parthenogenetically activated oocytes resided in their different calcium-spiking patterns (multiple and single spikes, respectively).

This explanation was then tested directly by preventing calcium spiking in oocytes fertilised in the presence of nocodazole prior to removal of the drug and spindle re-formation. When calcium spiking was prevented by addition of BAPTA-AM or exhaustion of calcium stores in Ca^{2+}-free medium, calcium spiking ceased in virtually all oocytes examined. When nocodazole was withdrawn subsequently, spindles re-formed, although they were not entirely normal in the presence of BAPTA-AM. Despite the re-formation of spindles, exit from M-phase did not occur in most oocytes. Unsurprisingly, buffering of calcium rises by BAPTA-AM was more efficient than simple calcium removal at achieving a complete inhibitory effect, probably due to the presence of traces of Ca^{2+} in culture medium components. As predicted from the proposition above, when calcium was then restored to the fertilised and calcium-depleted oocytes spiking resumed and exit from M-phase occurred.

Exit from M-phase requires net degradation of cyclin, and thus of MPF activity (Murray et al., 1989). The degradation of cyclin B in the mouse oocyte requires an intact spindle and proceeds continuously during metaphase II arrest at a moderate level, compensated by an ongoing synthesis of cyclin (Kubiak et al., 1993). Thus, activation by Ca^{2+} influx does not switch on cyclin degradation but rather increases it. In *Xenopus* eggs the Ca^{2+}-dependent inactivation of MPF activity at fertilisation is mediated by a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Lorca et al., 1991, 1993). However, in *Xenopus* eggs and early embryos, spindle depolymerisation does not arrest the cell cycle in M-phase (Clute and Masui, 1992; Gerhart et al., 1984). If a CaMKII is involved in mouse oocyte activation, our results suggest that it is only active during

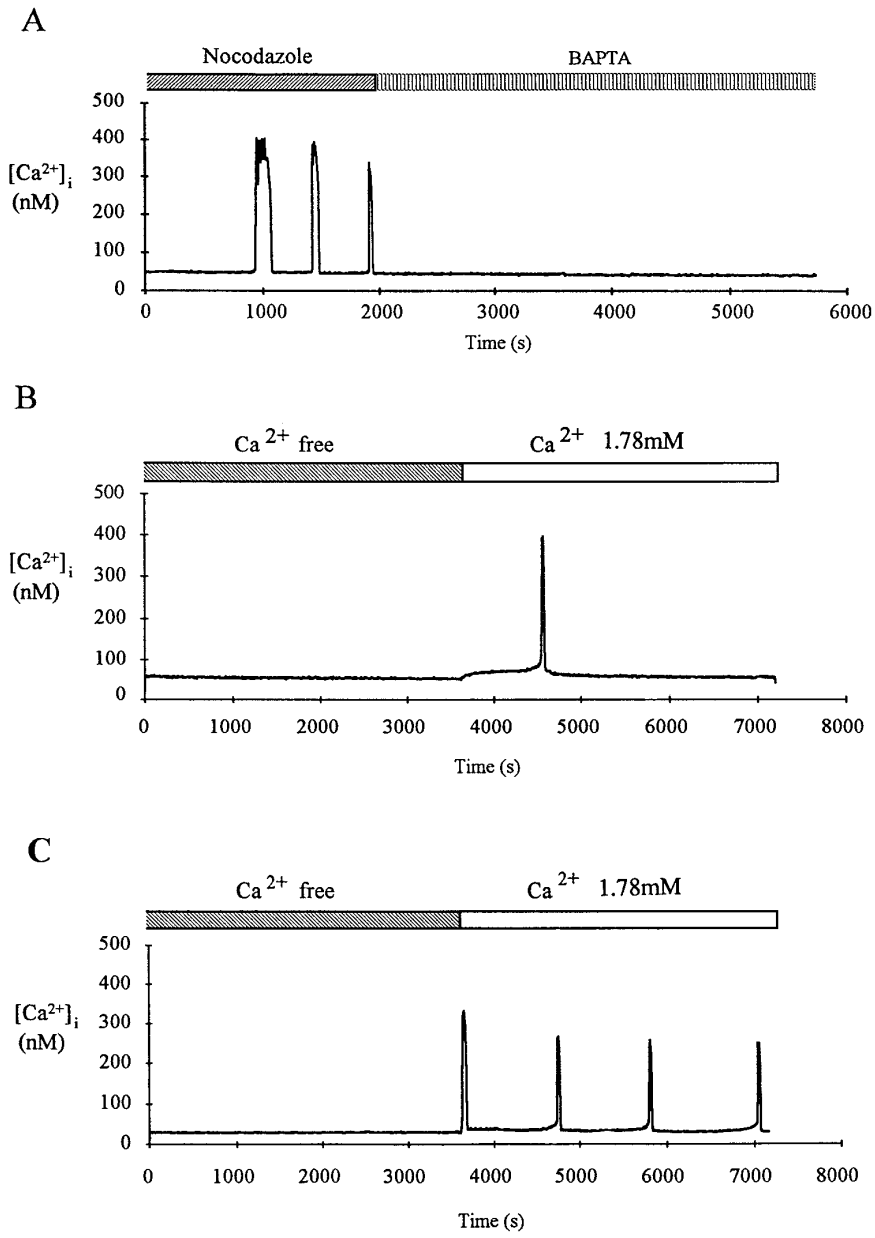

Fig. 6. (A) Oocytes washed out of nocodazole-containing fertilisation medium into medium supplemented with BAPTA-AM showed a complete cessation of calcium transients. (B,C) Likewise, when calcium was removed from the medium before spindle reformation had occurred, the calcium spiking ceased and the oocytes remained arrested in M-phase until external calcium was replaced, when spiking resumed and the cells passed into interphase.

(and/or shortly after) the calcium pulse and that all the other components of the cascade leading to cyclin degradation are only activated transiently. Alternatively, an intact spindle may be necessary to activate the CaMKII, since it seems to be also associated with the spindle (Ohta et al., 1990). Finally, these observations suggest that the 'memory of activation' observed in oocytes fertilised in the presence of nocodazole resides in the Ca^{2+}-spiking process and that the activation of the cyclin degradation machinery only takes place during (and/or shortly after) these Ca^{2+} spikes.

We thank Jacek Kubiak, Marie-Hélène Verlhac, Roger Morton, Tim Cheek and Mike Berridge for helpful suggestions, and Gérard Géraud, Brendan Doe and Christine Starr for technical support. This work was supported by the CNRS, and grants from the Ligue Nationale contre le Cancer, the Association pour la Recherche contre le Cancer and the Fondation pour la Recherche Médicale to B.M. and by a grant from the Agricultural and Food Research Council to M.H.J., Tim Cheek and Mike Berridge. N.J.W. was supported by fellowships from the Fondation pour la Recherche Médicale and from the Commission of the European Communities.

REFERENCES

Cheek, T. R., McGuinness, O. M., Vincent, C., Moreton, R. B., Berridge, M. J. and Johnson, M. H. (1993). Fertilisation and thimerosal stimulate calcium spiking patterns in mouse oocytes but by separate mechanisms. *Development* **119**, 179-189.

Clute, P. and Masui, Y. (1992). Development of microtubule-dependence of the chromosome cycle at the midblastula transition in Xenopus laevis embryos. *Dev. Growth Differ.* **34**, 27-36.

Colonna, R., Tatone, C., Malgaroli, A., Eusebi, F. and Mangia, F. (1989). Effects of protein kinase C stimulation and free Ca^{2+} rise in mammalian egg activation. *Gamete Res.* **24**, 171-183.

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.

de Pennart, H., Houliston, E. and Maro, B. (1988). Post translational modification of tubulin and the dynamics of microtubules in mouse oocytes and zygotes. *Biol. Cell* **64**, 375-378.

Draetta, G., Luca, F., Westerndorf, J., Brizuela, L., Ruderman, J. and Beach, D. (1989). cdc2 protein kinase is complexed with both cyclin A and B: Evidence for proteolytic inactivation of MPF. *Cell* **56**, 829-838.

Fraser, L. R. and Drury, L. (1975). The relationship between sperm concentration and fertilisation in vitro of mouse eggs. *Biol. Reprod.* **13**, 513-518.

Fraser, L. R. (1983). Mouse sperm capacitation assessed by kinetics and morphology of fertilisation in vitro. *J. Reprod. Fert.* **69**, 419-428.

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

Gallicano, G. I., Schwartz, S. M., McGaughey, R. W. and Capco, D. G. (1993). Protein kinase C, a pivotal regulator of hamster egg activation, functions after elevation of intracellular free calcium. *Dev. Biol.* **156**, 94-106.

Gerhart, J., Wu, M. and Kirschner, M. (1984). Cell cycle dynamics of an M-phase specific cytoplasmic factor in Xenopus laevis oocytes and eggs. *J. Cell Biol.* **98**, 1247-1255.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.

Kilmartin, J. V., Wright, B. and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* **93**, 576-582.

Kline, D. and Kline, K. 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.

Kubiak, J. Z., Weber, M., de Pennart, H., Winston, N. J. and Maro, B. (1993). The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF. *EMBO J.* **12**, 3773-3778.

Labbe, J.-C., Capony, J.-P., Caput, D., Cavadore, J.-C., Derancourt, J., Kaghdad, M., Lelias, J.-M., Picard, A. and Doree, M. (1989). MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO J.* **8**, 3053-3058.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lohka, M. J., Hayes, M. and Maller, J. (1988). Purification of maturation promoting factor, an intracellular regulator of early mitotic events. *Proc. Nat. Acad. Sci. USA* **85**, 3009-3013.

Lorca, T., Galas, S., Fesquet, D., Devault, A., Cavadore, J.-C. and Dorée, M. (1991). Degradation of the proto-oncogene product $p39^{mos}$ is not necessary for cyclin proteolysis and exit from meiotic metaphase: requirement for a Ca^{2+}-calmodulin dependent event. *EMBO J.* **10**, 2087-2093.

Lorca, T., Cruzalequi, F. H., Fesquet, D., Cavadore, J.-C., Méry, J., Means, A. and Dorée, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF activities upon the fertilization of Xenopus eggs. *Nature* **366**, 270-273.

Maro, B., Johnson, M. H., Pickering, S. J. and Flach, G. (1984). Changes in actin distribution during fertilisation of the mouse egg. *J. Embryol. Exp. Morphol.* **81**, 211-237.

Maro, B., Johnson, M. H., Webb, M. and Flach, G. (1986). Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma membrane. *J. Embryol. Exp. Morphol.* **92**, 11-32.

Moreton, R. B. (1991). Optical techniques and calcium imaging. In *Cellular Neurobiology, a Practical Approach* (ed. J. Chad and H. Wheal), pp. 205-222. IRL Oxford University Press.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**, 280-285.

Nasr-Esfahani, M., Johnson, M. and Aitken, R. J. (1990). The effect of iron and iron chelators on the in vitro block to development of the mouse preimplantation embryo: BAT6 a new medium for improved culture of mouse embryos in vitro. *Human Reprod.* **5**, 997-1003.

Newport, J. W. and Kirschner, M. W. (1984). Regulation of the cell cycle during early Xenopus development. *Cell* **37**, 731-742.

Nicholson, G. L., Yanagimachi, R. and Yanagimachi, H. (1975). Ultrastructural utilisation of lectin binding sites on the zonae pellucidae and plasm membranes of mammalian eggs. *J. Cell Biol.* **66**, 263-274.

Nurse, P. and Bissett, Y. (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. *Nature* **292**, 558-560.

Ohta, Y., Ohba, T. and Miyamoto, E. (1990). Ca^{2+}/calmodulin protein kinase II: Localization in the interphase nucleus and the mitotic apparatus of mammalian cells. *Proc. Nat. Acad. Sci. USA* **87**, 5341-5345.

O'Sullivan, A. J., Cheek, T. R., Moreton, R. B., Berridge, M. J. and Burgoyne, R. D. (1989). Localization and heterogeneity of agonist-induced changes in cytosolic calcium concentration in single bovine adrenal chromaffin cells from video imaging of fura-2. *EMBO J.* **8**, 401-411.

Shiina, Y., Kaneda, M., Marsuyama, K., Tanaka, K., Hiroi, M. and Doi, K. (1993). Role of the extracellular Ca^{2+} on the intracellular Ca^{2+} changes in fertilized and activated mouse oocytes. *J. Reprod. Fert.* **97**, 143-150.

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

Toyoda, Y., Yakogama, M. and Hoshi, T. (1971). Studies on the fertilization of mouse eggs in vitro. II. Effects of in vitro preincubation of spermatozoa on time of sperm penetration of mouse eggs in vitro. *Jap. J. Anim. Reprod.* **16**, 152-157.

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.

Vitullo, A. D. and Ozil, J-P. (1992). Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Dev. Biol.* **151**, 128-136.

Watanabe, N., Hunt, T., Ikawa, Y. and Sagata, N. (1991). Independent activation of MPF and cytostatic factor (Mos) upon fertilization of Xenopus eggs. *Nature* **352**, 247-248.

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

(Received 7 August 1994 - Accepted 14 September 1994)