Development 119, 179-189 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms

Timothy R. Cheek[1], Orla M. McGuinness[1,2,*], Caroline Vincent[2], Roger B. Moreton[1], Michael J. Berridge[1] and Martin H. Johnson[2]

[1]AFRC Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, CB2 3EJ, UK
[2]Department of Anatomy, Downing Street, Cambridge CB2 3DY, UK

*Author for correspondence

SUMMARY

Exposure of freshly ovulated mouse oocytes to a fertilising spermatozoon, thimerosal, Sr^{2+} or acetylcholine induced similar Ca^{2+} spiking responses. We propose that each of the four agents reduces the threshold for Ca^{2+} release from internal stores, but by different mechanisms.

All agents except thimerosal stimulated oocyte activation, but thimerosal caused dissassembly of the meiotic spindle and thus prevented progress into interphase. Dithiothreitol (DTT) completely blocked and reversed the spiking responses induced by thimerosal, but facilitated and accelerated those induced by spermatozoa, Sr^{2+} and acetylcholine. The stimulatory effect of DTT was not simply a consequence of progress into interphase, but was attributable, at least in part, to an enhancement of divalent cation entry, as measured by Mn^{2+} quench analysis of fura-2 in both fertilised and unfertilised oocytes. Possible mechanisms by which DTT might achieve its effects are discussed.

Key words: fertilisation, thimerosal, Ca^{2+}, Sr^{2+}, Mn^{2+}, dithiothreitol, oocytes, mouse

INTRODUCTION

The fertilisation of the mammalian oocyte is followed by a series of repetitive Ca^{2+} spikes (Cuthbertson et al., 1981; Igusa and Miyazaki, 1986; Miyazaki et al., 1986; Kline and Kline, 1992a). These Ca^{2+} spikes cause the release of cortical granules to induce the block to polyspermy, the reactivation of meiosis to initiate development (Kline and Kline, 1992b; Vincent et al., 1992) and, in the hamster, a series of corresponding hyperpolarising transients due to increases in K^+ conductance (Miyazaki and Igusa, 1981).

Two hypotheses have been advanced to explain how the spermatozoon might stimulate Ca^{2+} release. A sperm-associated molecule may interact with a receptor coupled to a G-protein in the oocyte membrane to activate phospholipase C, generate inositol 1,4,5-trisphosphate ($Ins P_3$) and thereby mobilise Ca^{2+} (Miyazaki, 1990; Jaffe, 1990). Activation of a G-protein in hamster oocytes by addition of 5-hydroxytryptamine (5-HT) or guanosine-5′-O-(3-thiotriphosphate) (GTPγS), or by the injection of $Ins P_3$, does induce a series of Ca^{2+} pulses, although their form differs from that reported at fertilisation (Miyazaki, 1988; Swann et al., 1989; Miyazaki et al., 1990). Moreover, both sperm- and GTPγS-induced Ca^{2+} transients are blocked by preinjection of guanosine-5′-O-(2-thiodiphosphate) (GDPβS; Miyazaki, 1988). However, this mechanism is unlikely to play a role in fertilisation because it is inhibited by phorbol ester, which has little effect on fertilisation (Swann et al., 1989; Miyazaki et al., 1990). Recently, a sperm ligand that is likely to bind to an oocyte integrin-like receptor has been described (Blobel et al., 1992; Tarone et al., 1993), but it is not clear that such a system would function through G-protein activation. Alternatively, the fertilising spermatozoon may introduce a soluble Ca^{2+}-releasing messenger into the oocyte cytoplasm after sperm:oocyte fusion (Dale et al., 1985; Stice and Robl, 1990; Swann, 1990).

The Ca^{2+} transients seem to be derived from internal Ca^{2+} stores since they can occur in the absence of external Ca^{2+} (Igusa and Miyazaki, 1983; Miyazaki, 1988), as can parthenogenetic activation via Ca^{2+} ionophore (Vincent et al., 1992). Depletion of Ca^{2+} stores by treatment with thapsigargin leads to reduction or inhibition of spikes (Kline and Kline, 1992a). However, refilling of internal pools by external Ca^{2+} is required; extended periods in Ca^{2+}-free medium lead to loss of Ca^{2+} transients. Transients may result from the sensitisation of a Ca^{2+}-induced Ca^{2+} release (CICR) system (Miyazaki, 1991; Swann, 1991). Recently, the selective sulphydryl reagent thimerosal has been shown to increase the sensitivity of CICR in unfertilised hamster (Swann, 1991) and mouse (Swann, 1992) oocytes, and its effects are blocked or reversed by dithiothreitol (DTT; Swann, 1992).

In this paper, we compare the Ca^{2+} transients induced in the mouse oocyte at fertilisation with those induced by

thimerosal. We propose that, while the latter may activate Ca^{2+} spiking by enhancing the sensitivity of InsP_3 receptors through a redox mechanism that is inhibited by DTT, the spiking that occurs after fertilisation is initiated by a separate mechanism, which is maintained or even accelerated by DTT. The origin of the DTT sensitivity of the spiking mechanism is investigated. The results show that DTT is able to increase the permeability of the oolemma to divalent cations in both fertilised and unfertilised oocytes and is also able to enhance known activators of Ca^{2+} spiking in oocytes. We suggest that DTT might enhance the transduction event that normally occurs at fertilisation to initiate both the influx of external Ca^{2+} and the onset of CICR. The nature of this transduction event and its potential constituent components are discussed.

MATERIALS AND METHODS

Oocytes

MF1 female mice (3-4 weeks; OLAC, Bicester, UK) and F_1 female mice (C57BL/10ScSn/Ola female × CBA/Ca/Ola male, bred in the laboratory) were superovulated by intraperitoneal injection of 5 or 10 i.u. of pregnant mare's serum gonadotrophin (PMS; Intervet, Cambridge, UK) followed 48 hours later by 5 or 10 i.u. of human chorionic gonadotrophin (hCG; Intervet). Unfertilised oocytes (12-13 hours post-hCG) were released from the oviduct into warmed medium H6+4 mg/ml bovine serum albumin (BSA; a Hepes-buffered form of modified T6 medium, Nasr-Esfahani et al., 1990). Cumulus cells were removed by brief exposure to hyaluronidase (0.1 mM; Sigma) and zonae pellucidae removed by exposure to alpha-chymotrypsin (0.001%; Sigma type II; Vincent et al., 1992). Oocytes were held in drops of H6+BSA under paraffin oil (FSA Laboratories, Loughborough, UK) in Falcon tissue culture dishes. All manipulations were carried out at 37°C on heated stages, pads or in incubators.

Spermatozoa were expelled from the vas efferentia and cauda epididymides of male CFLP mice into 1 ml of Whittingham's medium (Whittingham, 1971) containing 30 mg/ml BSA and incubated under oil for 1-3 hours at 37°C and 5% CO_2 to capacitate. Aliquots of 40-100 μl were taken for insemination of oocytes.

Oocytes were fertilised either by insemination of zona-free oocytes attached to the base of chambers on the warmed microscope stage (see below) or, when a longer interval between fertilisation and analysis of Ca^{2+} spiking was desired, in drops of T6+BSA medium under oil. These latter oocytes were then transferred to chambers.

Activation of oocytes parthenogenetically was achieved in three ways. (i) After a 5 to 15 minute incubation in H6 + BSA free of Ca^{2+}, oocytes were exposed to Ca^{2+} ionophore (ionomycin; 5 μM) in Ca^{2+}-free H6+BSA for 2 minutes. Full activation was confirmed by observation of polar body extrusion (Vincent et al., 1992). As DMSO was used as a solvent in the ionophore stock solution, an equivalent dilution of the highest concentration (0.1 or 0.25% DMSO) was used in control groups. After each treatment, oocytes were washed 5 times over a 30 minute period. The first three washes were performed in Ca^{2+}-free medium. (ii) Alternatively, after a 30 minute incubation in H6 + BSA containing 1.2 mM Ca^{2+}, oocytes were exposed to a solution of H6+BSA free of Ca^{2+} and phosphate but containing $SrCl_2$ ± DTT (at various concentrations as recorded under Results). (iii) In some experiments, after a 30 minute incubation in a solution of H6 + BSA containing 1.2 mM Ca^{2+}, oocytes were exposed to 50 μM acetylcholine in the same solution.

Microtubules and chromosomes in oocytes were stained in specially designed chambers as described in Maro et al. (1984). Cells were fixed at 37°C for 30 minutes in 3.7% formaldehyde in PBS in the presence of 0.5% Triton X-100 (Sigma) for extraction and were washed in phosphate-buffered saline (PBS). β-Tubulin was visualized with a rat monoclonal anti-tubulin antibody (Kilmartin et al., 1982) followed by rhodamine-labelled anti-rat IgG. Chromosomes were stained by incubation in Hoechst dye 33342 (10 μg ml^{-1} in PBS) for 30 minutes.

Cortical granule release was visualised by a procedure derived from that of Cherr et al. (1988). Oocytes were fixed in 3% paraformaldehyde in PBS for 30 minutes and then washed extensively in a blocking solution of 1 mg ml^{-1} BSA, 100 mM glycine and 0.2% sodium azide in PBS. To visualise exclusively the content of the cortical granules after extrusion, oocytes were not permeabilized. Oocytes were incubated in 10 μg ml^{-1} Lens culnaris agglutinin conjugated to fluorescein isothiocyanate (FITC-LCA; United States Biochemical Corporation) in blocking solution for 15 minutes and then washed extensively in the blocking buffer.

Intracellular divalent cation measurements

8-10 zona-free oocytes were washed and transferred to H6 + polyvinylpyrrolidone (PVP; 6 mg ml^{-1}) on a coverslip that had been precoated with concanavalin A (Con A; 0.2 mg ml^{-1} in PBS) and which formed the base of a metallic perfusion chamber (Moreton, 1991). Oocytes were then loaded with fura-2 acetoxymethlyester (2 μM; Molecular Probes) for 20 to 30 minutes and washed extensively with H6+PVP. The chamber was then placed in a well on the stage of a Nikon Diaphot TMD inverted epi-fluorescence microscope for imaging. Solutions were introduced via a system of continuous perfusion through the chamber maintained at 37°C.

Intracellular free divalent cation activity 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 stepper-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 lookup table to implement the formula of Grynkiewicz et al. (1985). The calculation was done in real time, to give a 'live' image of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which was updated every 80 msec, and smoothed by recursive filtering with a 200 msec time-constant to reduce the noise (for further details see O'Sullivan, 1989; Moreton, 1991). When both Ca^{2+} and Sr^{2+} were present in the incubation, the relative contribution of the two ions to changes in fluorescence was not established.

Mn^{2+} entry was followed by imaging the resulting quench in fura-2 fluorescence at 510 nm at a constant excitation wavelength of 360 nm. At this wavelength, fura-2 fluorescence is independent of $[Ca^{2+}]_i$ but fluorescense is still quenched by Mn^{2+} (Hallam et al., 1988).

In all cases, the live image was recorded continuously on video tape, and subsequently played back and re-digitised into a frame-store, using software written in the semper language (Synoptics Ltd.) to sample selected oocytes and to record and plot either mean $[Ca^{2+}]_i$ or fluorescence readings at regular time intervals. In most cases, data were sampled at 4 or 5 seconds intervals, but for more detailed analysis sampling could be speeded up to 0.8 seconds (eg. Fig. 2).

To determine whether fura-2 loaded oocytes had been fertilised, the coverslip was examined in the chamber under bright field for evidence of polar body extrusion. The coverslip was then removed, fixed in 4% formaldehyde for 20-40 minutes, rinsed in PBS, stained

with Hoechst 33258 (5 mg ml^{-1}; Sigma) for 10 minutes and mounted in Citifluor for assessment of sperm entry.

Materials

Acetylcholine, dithiothreitol, ionophore (ionomycin), thimerosal, $CaCl_2$, $MnCl_2$ and $SrCl_2$ were all from Sigma. Fura-2 AM was from Molecular Probes.

RESULTS

Ca^{2+} spiking induced by fertilisation or thimerosal

The Ca^{2+} responses of mouse oocytes to sperm and thimerosal are illustrated in Fig. 1. Following the insemination of spermatozoa into the chamber, the initiation of Ca^{2+} spiking in the first oocyte began after a delay of 7-15 minutes (Fig. 1A; *n*=63). The subsequent pattern of Ca^{2+} spikes varied considerably between oocytes and, in most experiments, some oocytes remained unfertilised; the Ca^{2+} levels in these served as controls for those in fertilised oocytes. In general, the first transient was broader and had a larger amplitude than the subsequent spikes as has been described by others (Kline and Kline, 1992a; Swann, 1992). In some oocytes, there was a gradual reduction in spike amplitude with time, as recorded in previous experiments on mouse (Cuthbertson et al., 1981) and hamster (Igusa et al., 1983) oocytes.

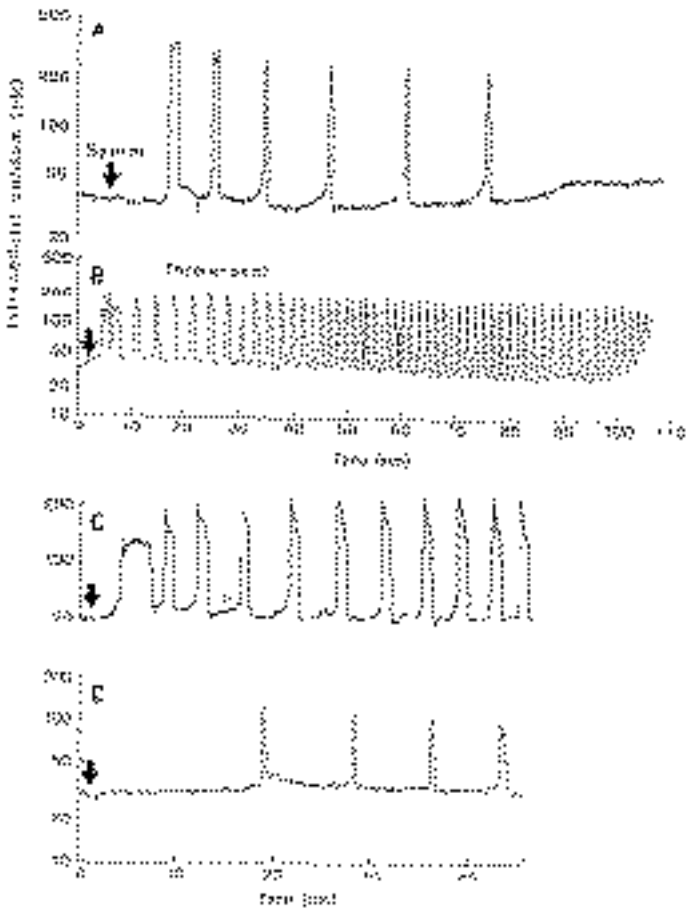

Fig. 1. Intracellular calcium spikes induced in mouse oocytes following addition of (A) sperm or (B-D) various concentrations of thimerosal. Spermatozoa were added at arrow (A) and remained throughout the experiment. The concentrations of thimerosal were (B) 100 μM, (C) 10 μM and (D) 1 μM. In B, thimerosal was applied initially at a concentration of 100 μM (B, arrow) but, after 5 minutes, the concentration was reduced to approximately 10 μM. These traces are representative of at least six oocytes studied at each thimerosal concentration. The asterisk illustrates a good example of the pacemaker rise in calcium that invariably precedes the onset of each calcium spike.

Addition of thimerosal to unfertilised oocytes produced a series of Ca^{2+} spikes which also usually began with a much broader spike (Fig. 1B,C). The threshold for the stimulatory action of thimerosal was approximately 1 μM (Fig. 1D), and both the amplitude and initial frequency of Ca^{2+} spiking were maximal between 10 and 20 μM (Fig. 1C). Further raising the thimerosal concentration to 100 μM had no marked effect on these initial parameters, but did accentuate the tendency for spikes to accelerate and the baseline to drift upwards (Fig. 1B). A characteristic feature of each spike is the gradual pacemaker rise in Ca^{2+} that precedes the rapid rising phase (see asterisk in Fig. 1C).

The characteristics of the individual spikes induced by fertilisation and thimerosal were remarkably similar with regard to their amplitude, width, rate of rise and rate of recovery (Table 1). In both cases, the rate of rise was always faster than the rate of recovery and, on average, each spike lasted for about 50 seconds with a relatively stable plateau which ended abruptly with the sudden onset of the recovery phase.

As has been recorded previously for hamster oocytes (Miyazaki et al., 1986), the first few transients following fertilisation appeared as waves in that the Ca^{2+} signal initiated at a focal point on one side of the oocyte and then spread to the opposite side at a rate of 47.5±6.06 μm sec^{-1} (*n*=10). This spatial organization is shown in Fig. 2A for the onset of the first Ca^{2+} transient following fertilisation. Subsequent chromosomal staining revealed that these waves initiated from the site of sperm entry. The first Ca^{2+} transient following thimerosal stimulation also appeared as a wave originating from a focal point but, with the resolution of our imaging system (5 ratio images sec^{-1}; see methods), the Ca^{2+} gradients did not appear as steep as those observed following fertilisation (Fig. 2B). For example, as the Ca^{2+}

Table 1. A quantitative analysis of the properties of calcium spikes induced by either fertilisation or thimerosal (10 μM)

	Rate of rise (nM/sec)	Rate of recovery (nM/sec)	Amplitude (nM)	Spike width (sec)
Thimerosal (*n*=9)	125±9.7	40±3.2	294±30.9	50±3.9
Fertilisation (*n*=11)	173±17.8	67±8.2	318±36.0	50±3.9

The typical shapes of the spikes that appear during fertilisation or treatment with 10 μM thimerosal are illustrated in Fig. 1. The third spike of the oscillatory train was chosen for this analysis. The rates of rise and recovery were obtained by measuring the maximum slopes of the rising and falling phases, respectively. The amplitude represents the difference between the peak and the resting values whereas the width was measured at the point where the spike was half maximal. Data show mean ± s.e.m.

wave spreads after fertilisation, an approximate 10-fold difference in $[Ca^{2+}]_i$ between the initiation site and the opposing pole of the oocyte can be detected, as judged by the false-colour (Fig. 2A, 5.6 seconds). In contrast, thimerosal-evoked Ca^{2+} waves appeared more diffuse and such large intracellular Ca^{2+} gradients were not observed (Fig. 2B). Thimerosal-evoked Ca^{2+} waves may reflect regional variations in the sensitivity of second messenger-mediated Ca^{2+} release (Rooney et al., 1991).

Ca^{2+} spiking in response to either fertilisation or thimerosal was very sensitive to changes in the concentration of external Ca^{2+}. Removal of Ca^{2+} abolished spiking whereas an increase of Ca^{2+} from the normal level of 1.2 mM to 2.4 or 12 mM resulted in acceleration (data not shown).

Thimerosal-induced spiking was reversible

In most oocytes, the spiking behaviour induced by fertilisation continued over the periods recorded (up to 3.5 hours post-insemination) regardless of whether spermatozoa were removed. By contrast, the thimerosal-induced spikes could be reversed if thimerosal was washed out of the bathing solution. In the experiment shown in Fig. 3A, removal of thimerosal (100 μM) at the end of the first spike resulted in the appearance of one additional spike, which was a consistent feature of the six oocytes analysed, followed by a complete cessation of the Ca^{2+} spikes observed normally in the continuous presence of this agent (cf. Fig. 1B with Fig. 3A). Upon the re-addition of thimerosal, a regular spiking pattern was restored rapidly (Fig. 3A), with no evidence of the initial, very broad spike observed normally when oocytes were first stimulated with thimerosal.

Dithiothreitol has opposite effects on the Ca^{2+} spiking induced by fertilisation or thimerosal

Thimerosal is a thiol reagent which probably acts by oxidizing sulphydryl groups, a process that can be reversed by dithiothreitol (DTT). The addition of DTT (8 mM) to oocytes stimulated in 20 μM thimerosal stopped the Ca^{2+} spiking immediately (Fig. 3C; n=10). Upon removal of DTT, regular spiking was restored after a delay. Note that the addition of DTT caused a small reversible depression of the baseline level of Ca^{2+} (Fig. 3C). In contrast to this inhibitory effect on thimerosal-induced Ca^{2+} spiking, DTT was found consistently to accelerate the spikes that develop following fertilisation (Fig. 3B; n=17). During the treatment with DTT, the period between spikes was approximately 100 seconds, which is very much shorter than the normal spike interval recorded following fertilisation (Fig. 1A). The acceleratory effect of DTT was reversible (Fig. 3B).

Effect of cortical granule release and oocyte activation on responsiveness to DTT

Fertilisation is associated with a number of changes to the organisation of the oocyte, any or all of which might influence the DTT responsiveness of Ca^{2+} spikes. The incorporation of the fusing spermatozoal membrane into the oolemma and the fusion of the cortical granules with the oolemma both result in a mosaic membrane structure with a composition differing from that prefertilisation (Wolf and Ziomek, 1983; Shapiro et al., 1981). The activation of the arrested meiotic state through the completion of metaphase to interphase is associated with changes in membrane properties, including the profile of functional ion channels in the oolemma (Day et al., 1990, 1991). DTT might, for example, stimulate Ca^{2+} influx through newly recruited ion channels. In an attempt to determine why DTT has a different effect on oocytes depending on whether they have been fertilised or treated with thimerosal, we examined whether or not thimerosal could induce some of the fertilisation events such as cortical granule release and oocyte activation.

Two techniques were used to assess whether or not cortical granule release occurred. A functional assay was provided by the acquisition of resistance by the zona pellucida to digestion by chymotrypsin - so called zona hardening. The data in Table 2 show that exposure to concentrations of thimerosal adequate to stimulate a train of Ca^{2+} spikes also causes zona hardening. A direct test for evidence of cortical granule discharge is to stain the exocytosed contents of the granules on the surface of the thimerosal-treated oocyte. Fig.

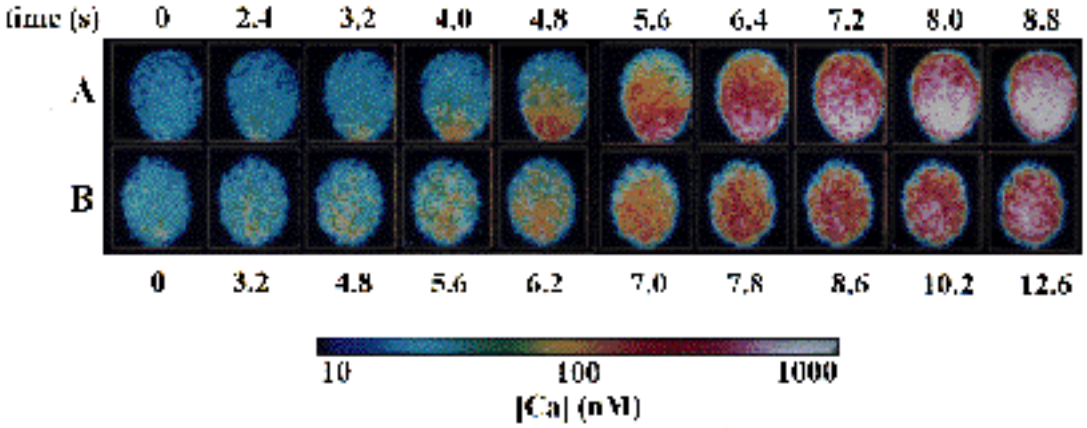

Fig. 2. The spatial organisation of the onset of the first intracellular calcium spike following addition of (A) sperm or (B) thimerosal (100 μM). False-colour pictures depict ratio images that were gathered at an effective rate of 5 ratio images sec^{-1}. The images at time=0 seconds show Ca^{2+} 0.8 seconds prior to the first detectable rise, and subsequent images were captured at the times indicated. In order to visualise the Ca^{2+} waves clearly, oocytes with relatively low Ca^{2+} propagation rates were chosen for this analysis.

4 confirms that cortical granules are indeed discharged. Of 24 control oocytes, only 3 (12%) showed evidence of cortical granule exocytosis, whilst of 40 oocytes exposed to 100 μM thimerosal for 5 minutes followed by a 10 to 30 minute wash, 39 (98%) showed exocytosis. These results make it unlikely that cortical granule exocytosis is responsible for the stimulatory effect of DTT on the fertilisation-induced train of spikes.

Although thimerosal induced cortical granule release, it did not activate oocytes. Thus, no polar body extrusion was observed in any of 34 control oocytes or any of 68 oocytes exposed to thimerosal (20-100 μM) for 30 minutes followed by a recovery period of 2 hours. Examination of oocytes immunocytochemically for tubulin and chromatin after exposure to 100 μM thimerosal for 11 to 40 minutes revealed that the spindle had been destroyed completely in 46/50 oocytes and severely disrupted in the remaining 4 oocytes. The oxidation of tubulin thiol groups is known to interfere with tubulin polymerisation and to be reversed by DTT (Kuriyama and Sakai, 1974). On neutralisation of the thimerosal with DTT, followed by an incubation in control medium, 30/41 oocytes had regenerated typical metaphase spindles (Fig. 5A) although in 11 of them the chromosomes were slightly dispersed from the metaphase plate. Of the remaining 11 oocytes, 10 had slightly abnormal spindles (Fig. 5C) and one lacked a spindle (Fig. 5B). An intact spindle is required for transition from M-phase to interphase (Maro et al., 1986), which therefore cannot occur during the period of exposure to thimerosal. Is it possible then that the passage from M-phase to interphase, causes fertilised oocytes to become sensitive to the stimulatory effect of DTT?

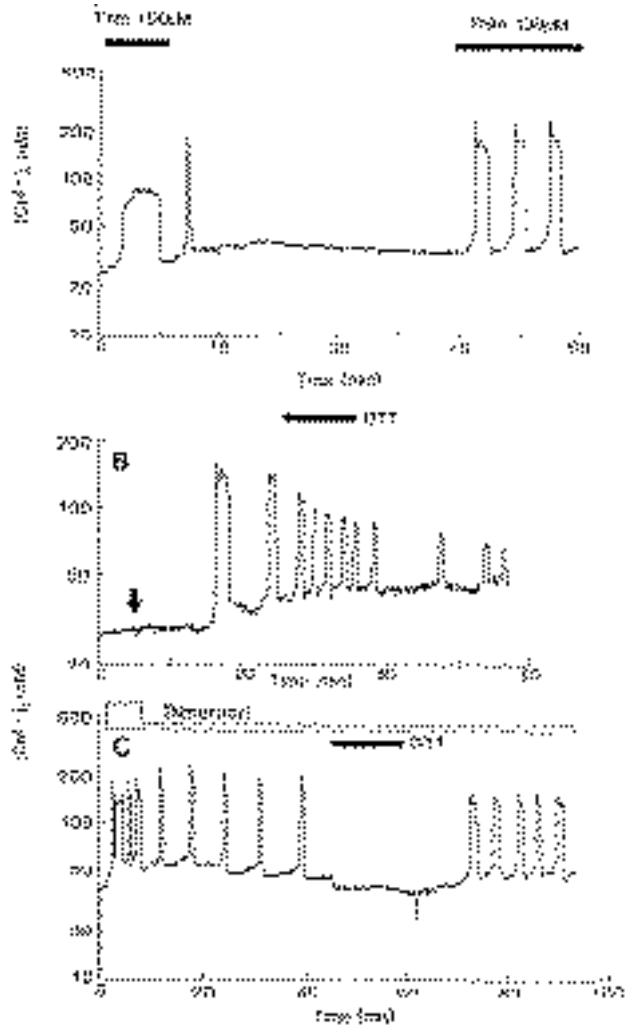

Fig. 3. The stimulatory effect of thimerosal (A, Thim) on calcium spiking is reversible, thimerosal (100 μM) being present during the period indicated by the solid bars. (B,C) The effect of dithiothreitol (DTT; solid bar) on the frequency of calcium spiking induced by (B) fertilization or (C) thimerosal. (B) Arrow marks the point of sperm addition. (C) Thimerosal was added initially at 100 μM (thick open bar) but the concentration was reduced after 6 minutes to 20 μM for the remainder of the experiment (thin open bar).

In an attempt to separate the reactivation of meiosis from other effects of sperm entry, oocytes were exposed to either spermatozoa (n=10) or Ca^{2+} ionophore (n=10), which activates development parthenogenetically by a single phasic release of Ca^{2+} from internal stores as well as inducing cortical granule release (Vincent et al., 1992). After culture for 1-2 hours to allow polar body extrusion and passage to interphase, the oocytes were loaded with fura-2. Both groups of oocytes, together with unfertilised oocytes, were then exposed to thimerosal to induce a train of spikes (Fig. 6). Superimposition of DTT resulted in the arrest of the spiking pattern in the unfertilised (Fig. 6C) and ionophore-activated (Fig. 6A) oocytes, but not in the fertilised oocytes (Fig. 6B). Thus, some change had apparently occurred in fertilised activated oocytes which was not evident in parthenogenetically activated oocytes, suggesting that the transition to interphase was not responsible for the changed response to DTT. It seemed possible that the fertilising sperm itself might introduce a component that conferred DTT sensitivity. Alternatively, a mechanism responsive to DTT might reside within the oocyte and be sensitised by sperm entry.

Sr^{2+} and DTT act synergistically to induce Ca^{2+} spiking

Activation by ionophore does not elicit the periodic Ca^{2+} spiking seen after sperm activation. An alternative approach to oocyte activation and the transition to interphase is to expose oocytes to Sr^{2+} (Fraser, 1987; Kline and Kline, 1992a), which yields spikes of divalent cations and thus more closely resembles fertilisation. We found that 8 mM

Table 2. Effect of exposure to thimerosal on zona reaction

Exposure of oocytes to thimerosal			% Oocytes with chymotrypsin resistant zonae
Concentration (μM)	Period (min)	No. of oocytes	
0	5	46	13
	30	29	17
10	5	16	25
	30	25	52
50	5	23	48
	30	21	100
100	5	72	100
	30	22	100

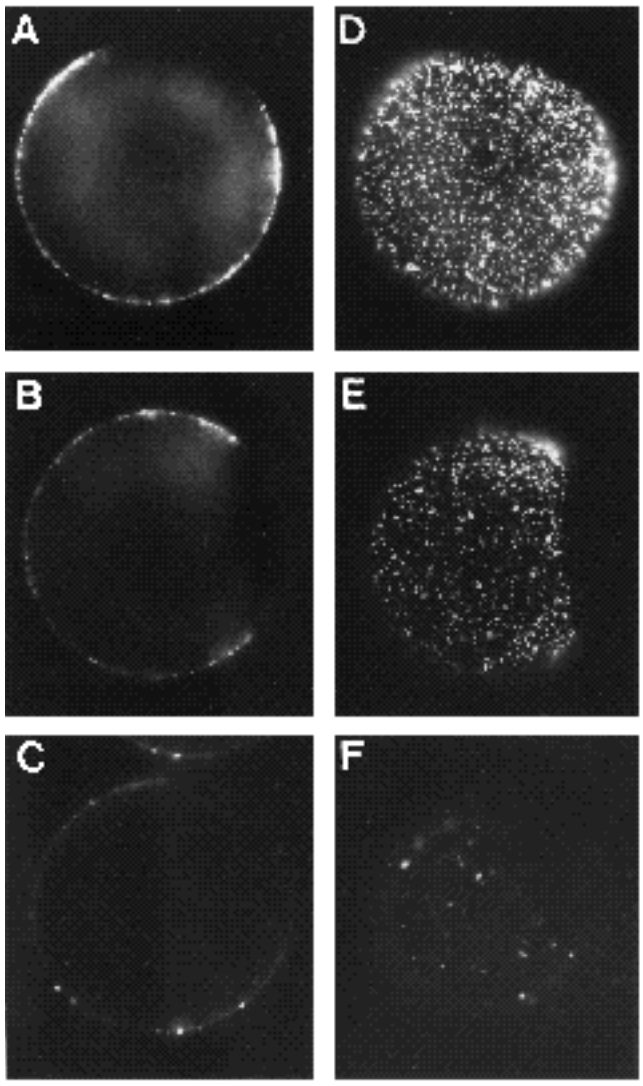

Fig. 4. Mouse oocytes stained for exocytosed cortical granule contents. (A,B,C) oocytes viewed in optical section and (D,E,F) oocytes viewed en face. (A,B,D,E) Oocytes exposed to 100 μM thimerosal for 5 minutes. (C,F) Control oocyte.

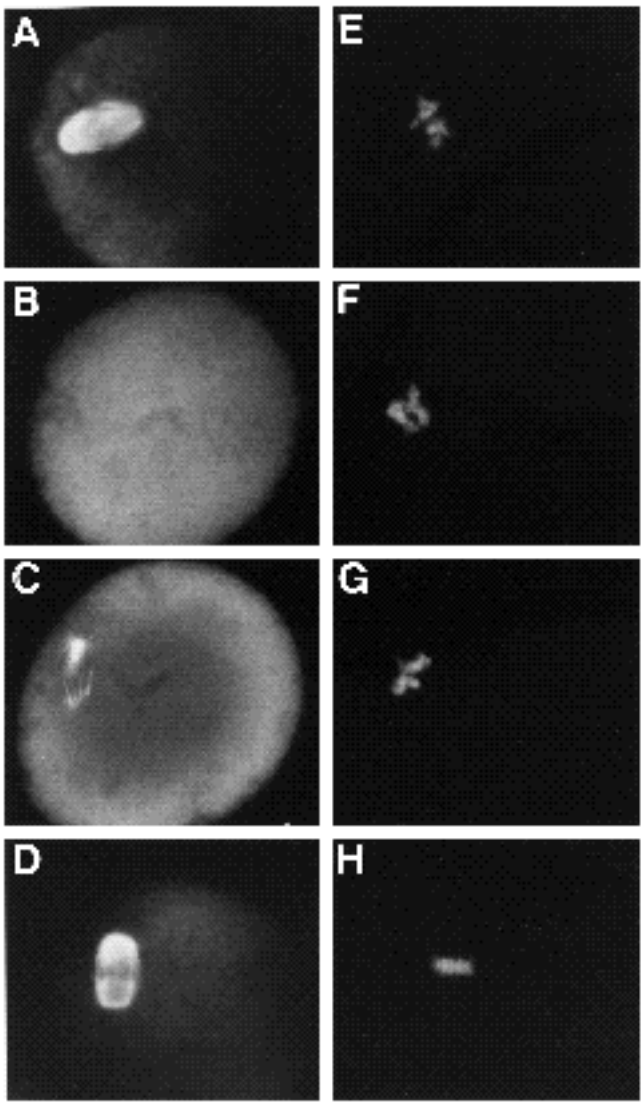

Fig. 5. Mouse oocytes stained for (A-D) tubulin and (E-H) chromatin. (A,E) Oocytes were exposed to 100 μM thimerosal for 5 minutes, then 20 μM thimerosal for 20 minutes, then washed free of thimerosal in control medium containing DTT for 15 minutes. (B,F,C,G) Oocytes exposed to thimerosal as for A,E, but fixed prior to wash out. (D,H) Control oocyte.

Sr^{2+} did not induce a train of spikes in unfertilised oocytes (*n*=18). However, superimposition of 8 mM DTT resulted in an immediate prolonged elevation of fluorescent signal (Fig. 7A; *n*=10). Conversely, oocytes that failed to respond to 8 mM DTT always responded to a subsequent addition of Sr^{2+}, again giving a maintained elevation of fluorescence (Fig. 7B; *n*=10). Sr^{2+} and DTT were acting in strict synergy and, by reducing their concentrations, it was possible to find a combination (4 mM Sr^{2+} and 1 mM DTT) that could reproduce a train of oscillations that resembled that seen at fertilisation in that the first spike was broader than subsequent spikes, and the spike frequency (and sometimes the amplitude) tended to fall with time (Fig 7C; *n*=61). All oocytes that showed a pulsatile response to Sr^{2+} or a prolonged elevation in their fluorescence also activated. In contrast, oocytes that were exposed to DTT in the presence of external Ca^{2+} concentrations up to 12 mM showed no pulsatile response and did not activate (Fig. 8A,B). In each instance, DTT elicited only a slight elevation of the basal intracellular Ca^{2+} concentration.

An alternative way of activating mammalian oocytes is to stimulate Ca^{2+} release via a receptor coupled G-protein (Miyazaki, 1988; Miyazaki et al., 1990). Acetylcholine (50 μM) triggered a train of Ca^{2+} spikes in mouse oocytes (Fig. 9; *n*=20). Superimposition of DTT consistently caused an acceleration in the train of spikes, reminiscent of its effect on the spikes following fertilisation (*n*=10).

Thus, in these experiments, the DTT sensitisation was evident when Sr^{2+} or acetylcholine substituted for a fertilising spermatozoon. These results suggest that a capacity to respond to DTT resides in the oocyte and does not require

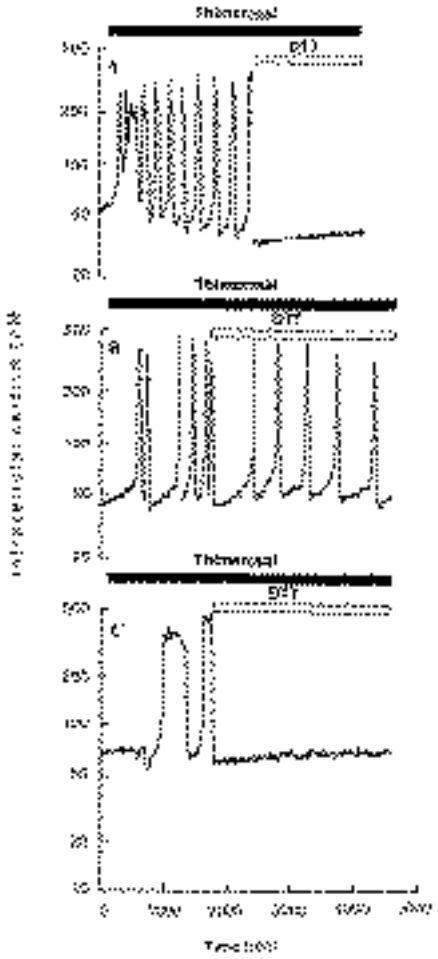

Fig. 6. The effect of dithiothreitol (DTT) on thimerosal-induced spiking in (A) ionophore- treated, (B) fertilised and (C) unfertilised mouse oocytes. Initially, oocytes were either inseminated with sperm or treated with 5 μM ionophore. Approximately 1 hour later the oocytes were incubated with fura-2 for 25 minutes. Changes in intracellular calcium ($[Ca^{2+}]_i$) were then monitored as shown. 100 μM thimerosal (solid bar) and 8 mM DTT (open bar) were added as indicated.

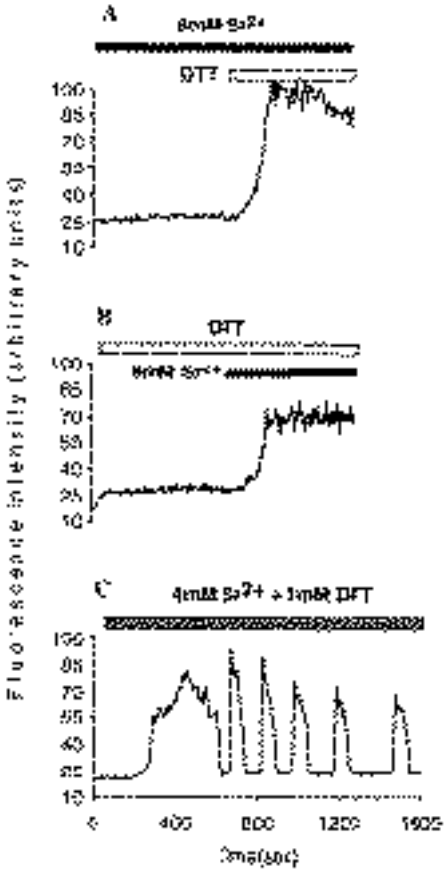

Fig. 7. Strontium (Sr^{2+}) and dithiothreitol (DTT) act synergistically to induce spiking in mouse oocytes. In traces A and B, 8 mM Sr^{2+} (solid bar) and 8 mM DTT (open bar) were added as indicated. In trace C, 4 mM Sr^{2+} and 1 mM DTT were added simultaneously (hatched bar).

a sperm for it to be revealed. What is the nature of this capacity and where in the oocyte does it reside?

Divalent cation influx increases in response to DTT

One possible effect of DTT might be to stimulate the influx of external Ca^{2+} across the oolemma. A more rapid refilling of internal stores would be expected to increase the frequency of Ca^{2+} spikes. It has been proposed but not demonstrated that fertilisation is accompanied by an increased influx of Ca^{2+} (Igusa and Miyazaki, 1983). To test the possibility that DTT increases divalent cation influx, unfertilised oocytes were loaded with fura-2 and placed in Mn^{2+} (0.1 and 0.5 mM), which also enters via divalent cation channels but quenches the fura-2 signal (Grynkiewicz et al., 1985). The fluorescence due to Mn^{2+} was recorded at a continuous excitation wavelength of 360 nm at which fura-2 emission is independent of $[Ca^{2+}]$ (Hallam et al., 1988). After establishing the basal rate of quench, DTT was added and the percentage increase in the Mn^{2+} quench rate calculated as 49±2 % (mean ± s.e.m. for 0.1 mM Mn^{2+}; n=5) and 50±10 % (mean ± s.e.m. for 0.5 mM Mn^{2+}; n=5) (Fig. 10A,B). Thus, DTT was indeed able to increase the rate of divalent cation influx into the oocytes.

The possibility that increased cation influx stimulated by DTT is responsible for the accleration in the fertilisation-induced train of spikes was examined more directly (Fig. 10C,D). Oocytes initially fertilised in Ca^{2+} showed the familiar train of spikes when Ca^{2+} was monitored by ratioing the fura-2 fluorescence at 340/380 nm (Fig. 10C; n=10). In a continuation of the same experiment, Ca^{2+} was replaced with 0.1 mM Mn^{2+} and the influx of Mn^{2+} was followed by monitoring the quench of fura-2 fluorescence at 360 nm. There was a rapid basal rate of quench (Fig. 10D). Superimposition of DTT resulted in a further increase in the rate of quench and the percentage increase was calculated as 76±37 % (mean±s.e.m. for 0.1 mM Mn; Fig. 10D). The DTT-induced acceleration of the fertilisation train might thus be explained by an enhanced influx of Ca^{2+} across the oolemma.

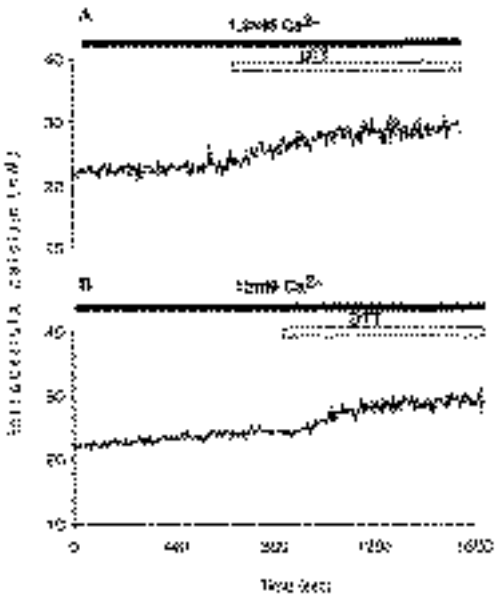

Fig. 8. The effect of 8 mM dithiothreitol (DTT) on intracellular calcium in the presence of (A) 1.2 mM (n=10) and (B) 12 mM (n=10) extracellular calcium.

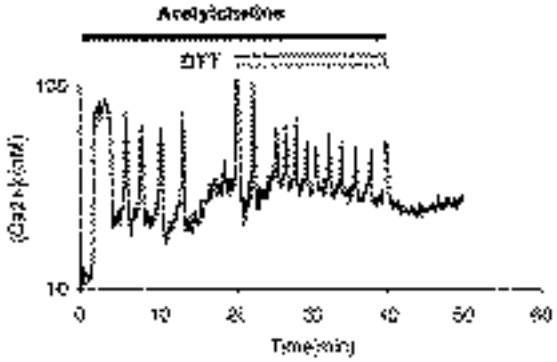

Fig. 9. The effect of 8 mM dithiothreitol (DTT; open bar) on the frequency of calcium spiking induced by acetylcholine (solid bar).

DISCUSSION

The response of mouse oocytes to sulphydryl reagents is paradoxical in that stimulation of Ca^{2+} spiking was observed following either oxidation (by thimerosal) or reduction (by dithiothreitol). The stimulatory effect of thimerosal on mouse oocytes was reversible and could also be inhibited by the reducing agent DTT. In contrast, when DTT was combined with other stimuli, it enhanced spiking, accelerating the spiking in oocytes activated by a spermatozoon or acetylcholine and acting synergistically with Sr^{2+} to induce spiking in quiescent oocytes. Unlike the other stimuli, thimerosal arrests oocytes in metaphase preventing completion of meiosis and progression into interphase. However, we have shown the arrested cell cycle does not account for the differential sensitivity of the oocytes to DTT since spikes induced by thimerosal in ionophore-activated, interphase oocytes are not accelerated by DTT. Moreover, DTT has stimulatory effects on cation influx in both fertilised (interphase) and unfertilised (meiotic) oocytes.

We have attempted to resolve the paradoxical response to sulphydryl reagents by proposing that thimerosal and DTT may be acting at two separate sites, which might correspond to the two processes that are thought to be activated at fertilisation. These two processes are, firstly, an increase in Ca^{2+} entry across the plasma membrane, which has been inferred from the observation that fertilisation caused the membrane potential to hyperpolarise (Igusa and Miyazaki, 1983). The second process, a ten-fold increase in the sensitivity of the internal Ca^{2+} stores to Ca^{2+}-induced Ca^{2+} release (CICR), was established by injecting pulses of Ca^{2+} of increasing amplitude to measure the triggering thresholds required in unfertilised and fertilised oocytes (Igusa and Miyazaki, 1983). Thus, the model proposed originally by Igusa and Miyazaki (1983) stressed the importance of the linkage of continuous Ca^{2+} influx to periodic release of intracellular Ca^{2+} via a process of CICR (Igusa and Miyazaki, 1983, 1986; Swann, 1991).

We argue below that thimerosal probably acts by mimicking the sensitisation of the internal release mechanism, whereas the stimulatory effects of DTT seem to depend, at least in part, upon the stimulation of Ca^{2+} influx events occurring at the plasma membrane.

Stimulatory effect of thimerosal

We have shown that the spatiotemporal characteristics of the thimerosal-induced Ca^{2+} signal in mouse oocytes are remarkably similar to those of the signal at fertilisation, suggesting that this thiol reagent is activating the same internal release mechanism by increasing the sensitivity of CICR (Swann, 1991; Miyazaki et al., 1992a; Carrol and Swann, 1992). The nature of the internal stores responsible for this regenerative release of Ca^{2+} has yet to be established, likely candidates for the receptors being those for ryanodine (Swann, 1992) or InsP_3 (Miyazaki et al., 1992b). The evidence for ryanodine receptors has come from studies on mouse oocytes, where ryanodine was found to release Ca^{2+} and appeared to enhance the sensitivity of oocytes to Ca^{2+} (Swann, 1992). The sensitivity of ryanodine receptors might be regulated by cyclic ADP ribose (cADPR) since the latter was able to potentiate Ca^{2+} release due to ryanodine in sea urchin eggs (Galione et al., 1991; McDougall et al., 1993). The Ca^{2+} spiking induced by thimerosal may result from a similar sensitisation of ryanodine receptors which have been shown to be potentiated by thiol reagents (Salama et al., 1992a,b).

Alternatively, an InsP_3 receptor may be involved in the sensitisation of hamster oocytes to Ca^{2+} injections (Swann, 1991; Carrol and Swann, 1992). Injection of InsP_3 into mammalian eggs triggers an all-or-none regenerative release of Ca^{2+} (Miyazaki, 1988; Peres, 1990; Peres et al., 1991), which might depend on the process of CICR as first proposed by Igusa and Miyazaki (1983). Studies on other cells have revealed that Ca^{2+} functions as a co-agonist on the InsP_3 receptor and thus contributes to an all-or-none release process through a positive feedback effect (Iino, 1990; Finch et al., 1991; Bezprozvanny et al., 1991; Iino and Endo, 1992). Moreover, studies on several cell types have shown that thiol oxidation can enhance Ca^{2+} release

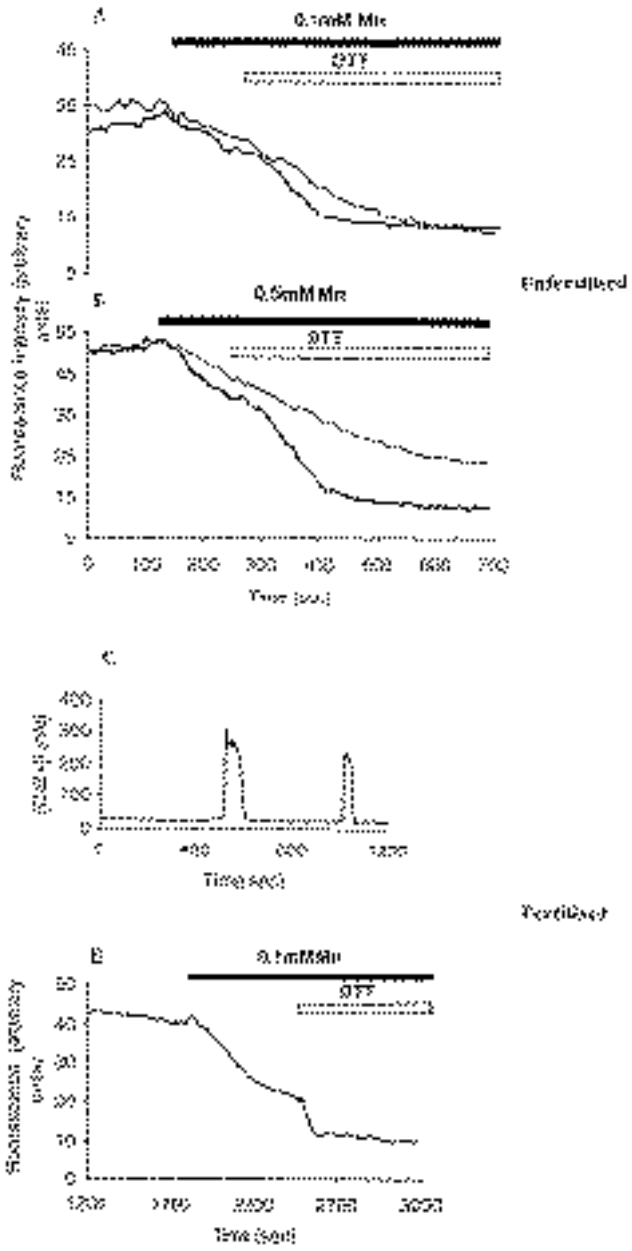

Fig. 10. The effect of 8 mM dithiothreitol (DTT; open bar) on the rate of divalent cation influx into (A,B) unfertilised and (C,D) fertilised mouse oocytes. Unfertilised oocytes were exposed to manganese (Mn^{2+}; solid bar) at concentrations of (A) 0.1 mM and (B) 0.5 mM. Mn^{2+} influx was followed by monitoring the resulting quench in fura-2 fluorescence at 360 nm (thick line). Representative traces of the basal rate of quench in the absence of DTT are shown for comparison (thin line). (C) One representative oocyte from a group that were fertilised in 1.2 mM Ca^{2+} and the resultant train of spikes monitored by ratioing the fura-2 fluorescence at 340/380 nm. (D) The same oocyte when, 30 min after fertilisation, fura-2 fluorescence was monitored at the Ca^{2+}-independent wavelength of 360 nm. The influx of 0.1 mM manganese (Mn^{2+}; solid bar) in the absence and presence of DTT (open bar) was followed by monitoring the resulting quench in fluorescence.

(Missiaen et al., 1991; Rooney et al., 1991; Bootman et al., 1992; Miyazaki et al., 1992a) and stimulate Ca^{2+} waves (Rooney et al., 1991). Since this sensitisation can be blocked by the InsP_3 receptor antibody 18A10, it has been argued that the regenerative release of Ca^{2+} by thimerosal can be brought about by InsP_3 receptors (Miyazaki et al., 1992b).

However, although it seems likely that thimerosal acts by oxidation of critical sulphydryl groups in either InsP_3 or ryanodine receptors to mimick the Ca^{2+} spiking that occurs at fertilisation, an oxidation-dependent mechanism cannot be involved normally, as fertilisation-induced spikes continue and are accelerated in the presence of DTT.

Stimulatory effect of DTT

Unlike thimerosal, DTT is not able to induce spiking by itself but it does have the remarkable capacity of enhancing all the known activators of Ca^{2+} spiking in mouse oocytes except thimerosal. Thus, addition of Sr^{2+} to mouse oocytes initiates repetitive spiking after a long delay (approximately 20 minutes; Kline and Kline, 1992b). The mode of action of Sr^{2+} is unknown but it may act as a surrogate in triggering CICR (Cognard and Raymond, 1985). Studies on frog skeletal muscle suggest that "the relative affinity of the internal Ca^{2+}-releasing stores appears to be Ba^{2+}>Sr^{2+}>Ca^{2+}" (Cognard and Raymond, 1985). The ability of Sr^{2+} to induce spiking was greatly potentiated by DTT through a mechanism that is associated with an enhanced divalent entry component as monitored by Mn^{2+} quenching of fura-2.

Indeed, stimulation of divalent cation entry is a consistent feature of the action of DTT both in unfertilised and fertilised oocytes. This increased rate of divalent cation entry would also account for the acceleration seen when DTT was superimposed on the train of spikes induced by either acetylcholine or a spermatozoon. Whether the stimulation of divalent entry is due to some direct action of DTT on a plasma membrane Ca^{2+} channel or due to the production of a second messenger, such as InsP_3, which in turn stimulates influx is unclear. However, the fact that DTT can act synergistically with Sr^{2+} to initiate a train of spikes indicates that this reducing agent can enhance the transduction event that leads to divalent cation influx and the onset of CICR.

Mechanisms linking sperm-oocyte interaction to the activation of Ca^{2+} spiking might also be sensitive to DTT. For example, the sperm may act via integrin receptors (Blobel et al., 1992; Tarone et al., 1993) or CD4-like molecules (Mori et al., 1992) on the oocyte to generate second messengers such as InsP_3. InsP_3 formation via integrin receptors may involve tyrosine kinase-linked mechanisms (Kornberg et al., 1991) coupled to phospholipase C-γ1 (Whitaker and Swann, 1993), a process known to induce Ca^{2+} oscillations in human neutrophils (Jaconi et al., 1991). Murine oocytes have a CD4/p56*lck* transducing system very similar to that found in T-cells (Mori et al., 1992) in which the oxidation of sulphydryls by N-ethylmaleimide inhibited the association between CD4 and pp56lck and the tyrosine phosphorylation of PLC-γ1 (Kanner et al., 1992). Thus, DTT may act by keeping critical cysteine residues reduced so that these proteins can interact with each other (Turner et al., 1990; Shaw et al., 1990). Inhibition of *v-src* by herbimycin A can be reversed by DTT, again suggesting that free SH-groups are essential for signal transduction through these non-

receptor tyrosine kinases (Vehara et al., 1989). Although the oocyte contains high levels of reducing power in the form of glutathione (10-20 mM; Calvin et al., 1986; Boerjan and de Boer, 1990; Nasr-Esfahani and Johnson, 1992), its use may require the activity of specific reducing enzymes, as this requirement has been found for the reduction of critical SH-groups involved in transcription factor-DNA and steroid hormone-receptor interactions (Xanthoudakis et al., 1992; Grippo et al., 1985). DTT may mimick the action of such enzymic factors to increase reducing conditions nonspecifically and thereby enhance the signalling events responsible for generating second messengers such as InsP_3.

What happens at fertilisation?

The experiments in this paper are consistent with the hypothesis originally proposed by Igusa and Miyazaki (1983) that Ca^{2+} spiking in oocytes depends upon the activation of two processes. Firstly, the case for an obligatory sensitisation of CICR for successful oocyte spiking is overwhelming, and this sensitisation can be achieved in a number of ways. Under normal conditions (or following acetylcholine stimulation), this sensitisation is probably mediated by a second messenger, of which InsP_3 or cADPR are likely candidates. Alternatively, the release channels (whether ryanodine or InsP_3) can be sensitised by oxidation of critical cysteine residues as occurs during the action of thimerosal or by the action of Sr^{2+} with its higher affinity for releasing internal Ca^{2+} stores. The second process is an increased influx of divalent cations, which may occur either in parallel with the increased sensitisation of CICR or may be linked to the latter, as might occur if the emptying of Ca^{2+} stores stimulates a secondary capacitative influx mechanism (Putney, 1986). Depletion of internal stores by thapsigargin enhances Ca^{2+} entry into mouse oocytes (Kline and Kline, 1992a), suggesting that such a capacitative mechanism exists.

The paradoxical action of the reagents DTT and thimerosal can be accommodated on the basis of the hypothesis that InsP_3 functions as a second messenger during fertilisation (Miyazaki et al., 1992b). The interaction between sperm and oocyte may initiate the production of InsP_3 through a mechanism that is enhanced by DTT. An increase in InsP_3 is then responsible for activating the two processes necessary to initiate Ca^{2+} spiking. It may act at the cell surface to promote the entry of external Ca^{2+} through a mechanism that remains to be defined. This enhanced entry of Ca^{2+} might then serve to sensitise the internal stores by virtue of increasing their Ca^{2+} content. However, such a sensitisation by Ca^{2+} seems unlikely, because DTT failed to induce spiking in unfertilised oocytes even when exposed to high concentrations of external Ca^{2+}. Alternatively the sensitisation of CICR may be achieved by InsP_3 diffusing in from the cell surface. Whatever the mechanism is, we expect that the signalling system will depend upon reduced sulphydryl groups to account for the stimulatory action of DTT.

Many thanks are due to Martin George and Brendan Doe for technical assistance. This work was supported by an AFRC grant to M. J. B., and a programme grant from the MRC to M. H. J. and Professor P. Braude. T. R. C. is a Royal Society University Research Fellow.

REFERENCES

Bezprozvanny, I., Watras, J. and Ehrlich, B. E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* **351**, 751-754.

Blobel, C. P., Wolfsberg, T. G., Turck, C. W., Myles, D. G., Primakoff, P. and White, J. M. (1992). A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. *Nature* **356**, 248-252.

Boerjan, M. L. and de Boer, P. (1990). The first cell cycle of zygotes of the mouse derived from oocytes and aged post-ovulation in vivo and fertilisation in vivo. In *Zygote Development after Delayed Fertilisation*. (ed. M. Boerjan). pp.17-33. Gradfisch bedrijf Ponsen and Looijen bv-Wageningen.

Bootman, M. D., Taylor, C. W. and Berridge, M. J. (1992). The thiol reagent, thimerosal evokes Ca^{2+} spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **267**, 25113-25119.

Calvin, H. I., Grosshans, K. and Blake, E. J. (1986). Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilised egg. *Gamete Res.* **14**, 265-275.

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**, 11196-11201.

Cherr, G. N., Drobnis, E. Z., and Katz, D. F. (1988). Localisation of cortical granule constituents before and after fertilisation in the hamster egg. *J. Exp. Zool.* **46**, 81-93.

Cognard, C. and Raymond, G. (1985). The Sr^{2+}-induced Ca^{2+}-release process and its implication in contractility of skeletal muscle of Rana ridibunda. *Proc. R. Soc. Lon. B* **224**, 489-509.

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

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.

Day, M. L., Pickering, S. J., Cook, D. I. and Young, J. A. (1990). Changes in ion channel populations during development in early mouse embryos. In *Exocrine secretion II* (ed. P.Y.D. Wong and J.A. Young). pp. 47-50.

Day, M.L., Pickering, S.J., Johnson, M.H. and Cook, D.I. (1991) Ion channels in the preimplantation embryo of the mouse. In *Ionic basis and energy metabolism of epithelial transport* (ed. I. Murakami, Y. Seo, A. Kuwahara and H. Watari), pp. 209-212. National Institute for Physiological Sciences, Okazaki, Japan.

Finch, E. A., Turner, T. J. and Goldin, S. M. (1991). Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* **252**, 443-446.

Fraser, L. R. (1987). Strontium supports capacitation and the acrosome reaction in mouse sperm and rapidly activates mouse eggs. *Gamete Res.* **18,** 363-374.

Galione, A., Lee, H. C. and Busa, W. B. (1991). Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates: Modulation by cyclic ADP-ribose. *Science* **253**, 1143-1146.

Grippo, J. F., Holmgren, A. and Pratt, W. B. (1985). Proof that the endogenous heat-stable glucocorticoid receptor-activating factor is thioredoxin. *J. Biol. Chem.* **260**, 93-97.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluoresence properties. *J. Biol. Chem.* **260**, 3440-3450.

Hallam, T. J., Jacob, R. and Merritt, J. E. (1988). Evidence that agonists stimulate bivalent-cation influx into human endothelial cells. *Biochem. J.* **255,** 179-184.

Igusa, Y. and Miyazaki, S-I. (1983). Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *J. Phys.* **340**, 611-632.

Igusa, Y. and Miyazaki, S-I. (1986). Periodic increase of cytoplasmic free calcium in fertilized hamster eggs measured with calcium-sensitive electrodes. *J. Phys.* **377**, 193-205.

Igusa, Y., Miyazaki, S-I. and Yamashita, N. (1983). Periodic hyperpolarizing responses in hamster and mouse eggs fertilized with mouse sperm. *J. Phys.* **340**, 633-647.

Iino, M. (1990). Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig Taenia caeci. *J. Gen. Physiol.* **95**, 1103-1122.

Iino, M. and Endo, M. (1992). Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. *Nature* **360,** 76-78.

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

Jaconi, M. E. E., Theler, J. M., Schlegel, W., Appel, R. D., Wright, S. D. and Lew, P. D. (1991). Multiple elevations of cytosolic-free Ca^{2+} in human neutrophils: initiation by adherence receptors of the integrin family. *J. Cell. Biol.* **112**, 1249-1257.

Kanner, S. B., Kavanagh, T. J., Grossmann, A., Hu, S-L., Bolen, J. B., Rabinovitch, P. S. and Ledbetter, J. A. (1992). Sulphydryl oxidation down-regulates T-cell signaling and inhibits tyrosine phosphorylation of phospholipase Cγ1. *Proc. Natl. Acad. Sci. USA* **89**, 300-304.

Kilmartin, J. V., Wright, B. and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a nonsecreting rat cell line. *J. Cell. Biol.* **22**, 699-707.

Kline, D. and Kline, J. T. (1992a). Thapsigargin activates a Ca^{2+} influx pathway in the unfertilised mouse oocyte and suppresses repetitive Ca^{2+} transients in the fertilised oocyte. *J. Biol. Chem.* **267**, 17624-17630.

Kline, D. and Kline, J. T. (1992b). Repetitive Ca^{2+} transients and the role of Ca^{2+} in exocytosis and cell cycle activation in the mouse oocyte. *Dev. Biol.* **149**, 80-89.

Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C. and Juliano, R. L. (1991). Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. *Proc. Natl. Acad. Sci. USA* **88**, 8392-8396.

Kuriyama, R. and Sakai, H. (1974). Role of tubulin -SH groups in polymerization to microtubules. *J. Biochem.* **76**, 651-654.

Maro, B., Johnson, M. H., Pickering, S. and Flach, G. (1984). Changes in actin distribution during fertilisation of the mouse egg. *J. Embryol. Exp. Morph.* **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. Morph.* **92**, 11-32.

McDougall, A., Gillot, I. and Whitaker, M. (1993). Thimerosal reveals calcium-induced calcium release in unfertilised sea urchin eggs. *Zygote* **1**, 35-42.

Missiaen, L., Taylor, C. W. and Berridge, M. J. (1991). Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature* **352**, 241-244.

Miyazaki, S-I. (1988). Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *J. Cell. Biol.* **106**, 345-353.

Miyazaki, S-I. (1990). Cell signalling at fertilization of hamster eggs. *J. Reprod. Fert. Suppl.* **42**, 163-175.

Miyazaki, S-I. (1991). Repetitive calcium transients in hamster oocytes. *Cell Calcium* **12**, 205-216.

Miyazaki, S-I. and Igusa, Y. (1981). Fertilization potential in golden hamster eggs consists of recurring hyperpolarizations. *Nature* **290**, 706-707.

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., Katayama, Y. and Swann, K. (1990). Synergistic activation by serotonin and GTP analogue and inhibition by phorbol ester of cyclic Ca^{2+} rises in hamster eggs. *J. Phys.* **426**, 209-227.

Miyazaki, S-I., Shirakawa, H., Nakada, K., Honda, Y., Yuzaki, M., Nakade, S. and Mikoshiba, K. (1992a). Antibody to the inositol trisphosphate receptor blocks thimerosal-enhanced Ca^{2+}-induced Ca^{2+} release and Ca^{2+} oscillations in hamster oocytes. *FEBS Lett.* **309** (2),180-184.

Miyazaki, S-I.,, Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. (1992b). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilised hamster oocytes. *Science* **257**, 251-255.

Moreton, R. B. (1991). Optical techniques and Ca^{2+} imaging. In *Cellular Neurobiology, a Practical Approach* (ed. J. Chad and H. Wheal) pp. 205-222. IRL Oxford University Press.

Mori, T., Gou, M. W., Yoshida, H., Saito, S. and Mori, E. (1992). Expression of the signal transducing regions of CD4-like and lck genes in murine oocyte. *Biochem. Biophys. Res. Comm.* **82**, 527-533.

Nasr-Esfahani, M., Johnson, M. H. 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 oocytes in vitro. *Human Reprod.* **5**, 997-1003.

Nasr-Esfahani, M. H. and Johnson, M. H. (1992). Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing in vivo and in vitro. *Human Reprod.* **7**, 1281-1290.

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.

Peres, A. (1990). $InsP_3$- and Ca^{2+}-induced Ca^{2+} release in single mouse oocytes. *FEBS Lett.* **275**, 213-216.

Peres, A., Bertollini, K. and Racca C. (1991). Characterization of Ca^{2+} transients induced by intracellular photorelease of $InsP_3$ in mouse ovarian oocytes. *Cell Calcium* **12**, 457-465.

Putney, J. W. (1986). A model for receptor regulated calcium entry. *Cell Calcium* **7**, 1-12.

Rooney, T. A., Renard, D. C., Sass, E. J. and Thomas, A. P. (1991). Oscillatory cytosolic calcium waves independent of stimulated inositol 1,4,5-trisphosphate formation in hepatocytes. *J. Biol. Chem.* **266**, 12272-12282.

Salama, G., Abramson, J. J. and Pike, G. K. (1992a). Sulphydryl reagents trigger Ca^{2+} release from the sarcoplasmic reticulum of skinned rabbit psoas fibres. *J. Physiol.* **454**, 389-420.

Salama, G., Nigam, N., Shome, K., Lagenaur, C. and Zaidi, N. F. (1992b) Ryanodine affinity chromatography purifies 106kD Ca^{2+} release channels from skeletal and cardiac sarcoplasmic reticulum. *Cell Calcium* **13**, 635-647.

Shapiro, B. M., Schackmann, R. W. and Gabel, C. A. (1981). Molecular approaches to the study of fertilization. *Ann. Rev. Biochem.* **50**, 815-843.

Shaw, A. S., Chalupny, J., Whitney, J. A., Hammond, C., Amrein, K. E., Kavathas, P., Sefton, B. M. and Rose, J. K. (1990). Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56lck tyrosine protein kinase. *Mol. Cell. Biol.* **10**, 1853-1862.

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

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

Swann, K. (1991). Thimerosal causes calcium oscillations and sensitizes calcium-induced calcium release in unfertilized hamster eggs. *FEBS Lett.* **278**, 175-179.

Swann, K. (1992). Different triggers for Ca^{2+} oscillations in mouse oocytes involve a ryanodine-sensitive Ca^{2+} store. *Biochem. J.* **287**, 79-84.

Swann, K., Igusa, Y. and Miyazaki, S-I. (1989). Evidence for an inhibitory effect of protein kinase C on G-protein-mediate repetitive calcium transients in hamster eggs. *EMBO J.* **8**, 3711-3718.

Tarone, G., Russo, M. A., Hirsch, E., Odorisio, T., Altruda, F., Silengo, L. and Siracusa, G. (1993). Expression of β1 integrin complexes on the surface of unfertilized mouse oocyte. *Development* **117,** 1369-1375.

Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. D., Perlmutter, R. M., and Littman, D.R. (1990). Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell* **60**, 755-765.

Vehara, Y., Fukazawa, H., Murakami, Y. and Mizuno, S. (1989). Irreversible inhibition of v-src tyrosine kinase activity by herbimycin A and its abrogation by sulphydryl compounds. *Biochem. Biophys. Res. Comm.* **163**, 803-809.

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

Whitaker, M. and Swann, K. (1993). Lighting the fuse at fertilisation. *Development* **117**, 1-12.

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

Wolf, D. E. and Ziomek, C. A. (1983). Regionalisation and lateral diffusion of membrane proteins in unfertilized and fertilized mouse eggs. *J. Cell Biol.* **96**, 1786-1790.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y. E. and Curran, T. (1992). Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J.* **11**, 3323-3335.

(Accepted 28 May 1993)