

# A direct measurement of increased divalent cation influx in fertilised mouse oocytes

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## SUMMARY

On fertilisation of mouse oocytes, the fusing spermatozoon triggers a series of repetitive calcium ( $\text{Ca}^{2+}$ ) spikes. These  $\text{Ca}^{2+}$  spikes seem to be necessary for successful progression through the cell cycle and are regulated in a cell-cycle-dependent manner. The spikes appear to require the linkage of continuous  $\text{Ca}^{2+}$  influx to the periodic release of  $\text{Ca}^{2+}$  from intracellular stores by a process of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. The precise role of  $\text{Ca}^{2+}$  influx was explored using the manganese ( $\text{Mn}^{2+}$ )-quench technique to monitor unidirectional cation influx into single mouse oocytes. There was a marked stimulation of cation influx associated closely with the upswing of the first and subsequent fertilisation  $\text{Ca}^{2+}$  spikes. A smaller but significant increase in the rate of cation influx persisted in the interspike period in fertilised oocytes. Spike-associated entry was not as apparent in oocytes stimulated to spike repetitively by thimerosal or acetylcholine application. Instead, there was a continuous increase in cation influx underlying  $\text{Ca}^{2+}$  spiking which commenced with the onset of the first spike. Using the specific microsomal inhibitor thapsigargin and the  $\text{Ca}^{2+}$

ionophore ionomycin, we found evidence for a capacitative entry mechanism in mouse oocytes. We propose that the persistent influx of  $\text{Ca}^{2+}$  observed in response to all stimuli examined is controlled by a capacitative mechanism and sets the frequency of spiking by determining the time taken to refill the internal stores to a point where they are again sensitive enough to initiate the next spike.

Abbreviations:  $\text{Ca}^{2+}$ , calcium;  $\text{Mn}^{2+}$ , manganese;  $\text{Sr}^{2+}$ , strontium;  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_o$ , extracellular free  $\text{Ca}^{2+}$  concentration;  $\text{InsP}_3$ , inositol 1,4,5-trisphosphate; Ry, ryanodine; CICR,  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release; BAPTA, 1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PVP, polyvinylpyrrolidone; VOC, voltage-operated  $\text{Ca}^{2+}$  channel

Key words: divalent cation, calcium, manganese, mouse, oocyte, fertilisation, thapsigargin, spermatozoon

## INTRODUCTION

On fertilisation of mammalian oocytes, gamete fusion triggers a series of repetitive calcium ( $\text{Ca}^{2+}$ ) spikes (Cuthbertson and Cobbold, 1985; Miyazaki, 1991; Sun et al., 1992; Fissore et al., 1992; Taylor et al., 1992; Cheek et al., 1993). In response to this signal, the oocyte completes meiosis with the extrusion of the second polar body, entry into interphase and cortical granule exocytosis (Kline and Kline, 1992a; Whitaker and Swann, 1993). The development of the zygote ensues. The frequency of  $\text{Ca}^{2+}$  spiking can affect the speed of progression into the cell cycle (Vitullo and Ozil, 1992). More recently, it has been shown that these repetitive  $\text{Ca}^{2+}$  spikes are regulated in a cell-cycle-dependent manner (Jones et al., 1995). Normally, the spikes cease at around the time of pronuclear formation, but they continue indefinitely in metaphase-II-arrested oocytes.

The mechanism by which sperm fusion initiates the train of spikes is unknown. It is thought that the sperm must somehow

sensitise intracellular  $\text{Ca}^{2+}$ -release channels, probably the inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) receptor (Miyazaki et al., 1993; Ayabe et al., 1995). In addition to a sensitisation of the  $\text{Ca}^{2+}$ -release channels, Igusa and Miyazaki (1983) suggested that a continuous influx of  $\text{Ca}^{2+}$  is needed to maintain a train of  $\text{Ca}^{2+}$  spikes. In hamster and mouse oocytes, a reliance on external  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_o$ , is borne out by the fact that on removal or chelation of  $[\text{Ca}^{2+}]_o$  with BAPTA, spiking quickly stops or is reduced markedly in frequency (Igusa and Miyazaki, 1983; Kline and Kline, 1992a). More support for the idea that activation of  $\text{Ca}^{2+}$  influx is involved in mediating an oscillatory train of  $\text{Ca}^{2+}$  spikes, comes from the observation that repetitive  $\text{Ca}^{2+}$  spiking can be induced in mouse oocytes using dithiothreitol (DTT) and strontium ( $\text{Sr}^{2+}$ ) (Cheek et al., 1993). DTT has been shown to stimulate divalent cation influx across the oolemma (Cheek et al., 1993) and would facilitate the entry of  $\text{Sr}^{2+}$  which effectively mimics the action of  $\text{Ca}^{2+}$  and  $\text{InsP}_3$  to stimulate  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release, CICR (Cognard and Raymond, 1985).

In this study, the relationship between  $\text{Ca}^{2+}$  entry and spiking was explored using the manganese ( $\text{Mn}^{2+}$ )-quench technique (Hallam et al., 1988) to monitor unidirectional cation influx into single mouse oocytes. The specific microsomal inhibitor thapsigargin and the  $\text{Ca}^{2+}$  ionophore ionomycin were used to determine whether or not there is a capacitative  $\text{Ca}^{2+}$  influx pathway in mouse oocytes. For the first time, an increased divalent cation entry linked to the periodic release of  $\text{Ca}^{2+}$  from the internal stores of fertilised mouse oocytes has been demonstrated directly.

## MATERIALS AND METHODS

### Oocytes and spermatozoa

MF1 female mice (3-4 weeks; OLAC, Bicester, UK) 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 zona 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 fertilization medium (Whittingham, 1968) containing 30 mg/ml BSA and incubated under oil for 1-3 hours at 37°C and 5%  $\text{CO}_2$  to capacitate. Aliquots of 40-100  $\mu\text{l}$  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  $\text{Ca}^{2+}$  spiking was desired, in drops of T6+BSA medium under oil. These latter oocytes were then transferred to chambers.

### Intracellular divalent cation measurements

8-20 zona-free oocytes were washed and transferred to H6 + polyvinylpyrrolidone (PVP; 6 mg/ml) on a coverslip that had been precoated with concanavalin A (Con A; 0.2 mg/ml in PBS) and which formed the base of a metallic perfusion chamber (Moreton, 1991). Oocytes were then loaded with fura-2 acetoxymethyl ester (2  $\mu\text{M}$ ; Molecular Probes) for 30-40 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  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) activity alone was imaged through a Nikon CF-Fluor 20 $\times$  objective and intensified CCD camera (Extended ISIS, Photonic Science, Robertsbridge, UK), by following changes in 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 using lookup tables to implement the formula of Grynkiewicz et al. (1985):

$$[\text{Ca}^{2+}]_i = \frac{K_d R - R_{\min}}{R_{\max} - R} \frac{S_{f2}}{S_{b2}},$$

where  $K_d$  is the dissociation constant for fura-2/ $\text{Ca}^{2+}$  (224 nM),  $R$  is

the intensity ratio for fluorescence at the two chosen wavelengths,  $R_{\min}$  and  $R_{\max}$  are ratios at zero and saturating  $[\text{Ca}^{2+}]_i$ , respectively, and  $S_{f2}/S_{b2}$  is the ratio of excitation efficiencies for free and bound fura-2 at the higher of the two wavelengths. The calculation was done in real time, 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 et al., 1989; Moreton, 1991).

All ratios were determined empirically using the in vitro calibration method (Moreton, 1992), by measuring the fluorescence intensities of bulk solution fura-2 free acid in  $\text{CaCl}_2/\text{EGTA}$  buffers prepared in a 100 mM KCl medium with 10 mM EGTA and 10 mM MOPS at pH 7.2. This calibration method may underestimate  $[\text{Ca}^{2+}]_i$  by 25-30%, because the lack of polarity and viscosity in the bulk solutions can lead to an overestimate of both  $R_{\min}$  and  $R_{\max}$  by about 15% (Moreton, 1992).

$\text{Mn}^{2+}$  entry was monitored in parallel with changes in  $[\text{Ca}^{2+}]_i$  by imaging the resulting quench in fura-2 fluorescence at 510 nm excited alternately at 340 and 360 nm. At 360 nm fura-2 fluorescence is independent of  $[\text{Ca}^{2+}]_i$  and any decrease in fluorescence is due only to  $\text{Mn}^{2+}$  entry (Hallam et al., 1988) and this value was verified in situ for mouse oocytes (see Fig. 2). An indication of  $[\text{Ca}^{2+}]_i$  is given by the 340 nm wavelength alone, or the 340/360 nm ratio which eliminates artifacts due, for example, to changes in the dye distribution. The imaging software was used to switch the display rapidly between ratio and single-wavelength modes, at regular time intervals and with a switching time of 40 msec (one video frame).

In all cases, the live image was recorded continuously on video tape, and subsequently played back and redigitised into a frame-store, using software written in the semper language (Synoptics Ltd.) to sample selected oocytes and to record and plot either mean  $[\text{Ca}^{2+}]_i$  or fluorescence readings at regular time intervals. Data points were sampled from the continuous record every 5 seconds when  $[\text{Ca}^{2+}]_i$  alone had been monitored and every 2 seconds when  $\text{Mn}^{2+}$  entry had been monitored in parallel with changes in  $[\text{Ca}^{2+}]_i$ .

### Data analysis

A Student's  $t$ -test was used to compare  $\text{Mn}^{2+}$  quenching rates among batches of oocytes loaded with fura-2 and imaged on the same coverslip (spiking versus quiescent; fertilised versus unfertilised etc.) The final values are expressed as a ratio and the significance of any difference indicated.

### Depletion of intracellular $\text{Ca}^{2+}$ stores with thapsigargin

Thastrup et al. (1990) introduced thapsigargin into widespread use and it is normally effective at concentrations below 1  $\mu\text{M}$ . As previously observed by Kline and Kline (1992b), mouse oocytes, like *Xenopus* oocytes (Peterson and Berridge, 1994), are relatively insensitive to thapsigargin. Therefore, 20  $\mu\text{M}$  thapsigargin was used routinely to deplete the intracellular  $\text{Ca}^{2+}$  stores. Dimethyl sulphoxide was used to dissolve the thapsigargin; after dilution, the final concentration of dimethylsulphoxide was 0.1%.

### Materials

Acetylcholine, thimerosal, ionomycin,  $\text{CaCl}_2$  and  $\text{MnCl}_2$  were all from Sigma. Thapsigargin was from Calbiochem. Fura-2 AM was from Molecular Probes.

## RESULTS

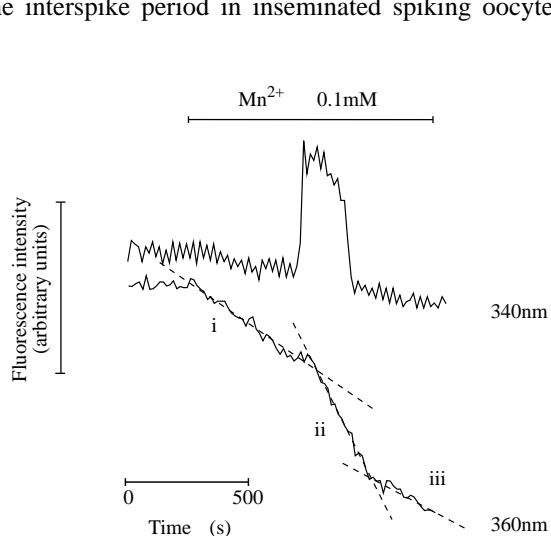
### Divalent cation influx across the oolemma increases after fertilisation

In order to monitor unidirectional cation influx into single mouse oocytes we employed the  $\text{Mn}^{2+}$  quench technique (Hallam et al., 1988). Quenching of fura-2 fluorescence by  $\text{Mn}^{2+}$  was monitored at the isosbestic wavelength of 360 nm.

There was a basal rate of fluorescence quenching due to  $Mn^{2+}$  entry in control oocytes and in inseminated oocytes that had not been fertilised. Addition of  $Mn^{2+}$  alone had no effect on the 340/380 nm ratio. In 25 oocytes (6 experiments) that spiked in response to insemination,  $Mn^{2+}$  quenching was examined during the period leading up to the first spike. There was no clear evidence for any stimulation of quench rate during this latent period (Fig. 1, rate i). However, a marked stimulation of fluorescence quenching coincided with the initiation of the first spike in 21 of the 25 oocytes that spiked as shown in Fig. 1. The stimulated rate of fluorescence quenching was closely associated with the upstroke of the spike (Fig. 1, rate ii). In 4/25 oocytes, no stimulated rate of fluorescence quench was detected either before, during or after the first spike (results not shown) but, in these cases, the basal rate of fluorescence quenching was very high and comparable to the maximum rate of fluorescence quenching measured in the other 21 oocytes. In one representative experiment, 9/11 oocytes showed a stimulated quench rate associated with the first spike and this rate was, on average, 1.9-fold greater than that measured in the preceding latent period ( $P=0.002$ ).

The faster quench rate observed during the spike was not maintained, but slowed down following the downstroke of the spike (Fig. 1, rate iii). Even though the quench rate was reduced after the spike, it usually remained higher than the basal rate of quenching measured in the preceding latent period (Fig. 1; rate iii versus rate i). Sometimes the quench rate appeared to slow down after the first spike, to a rate comparable to, or slower than, the basal rate, but this may reflect a saturation of the quenching as the intracellular  $[Mn^{2+}]$  increases.

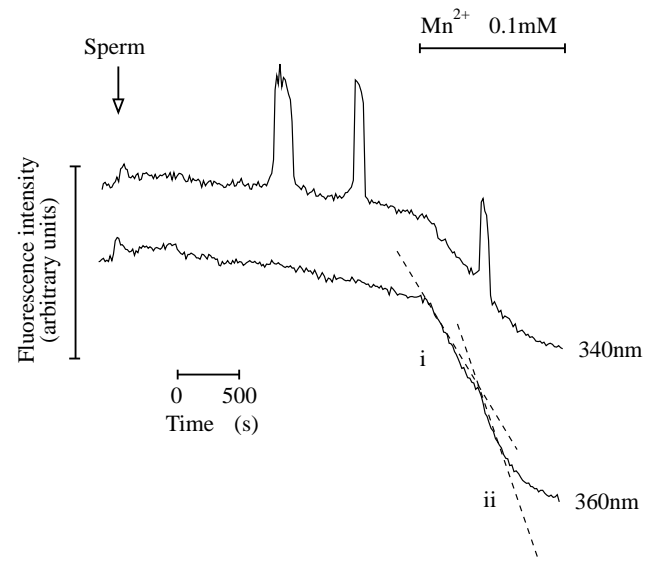
In order to study the quench rate during the interspike interval in more detail, we studied oocytes where  $Mn^{2+}$  was added during this interval such that the concentration of fura-2 was not rate-limiting. The rate of fluorescence quenching in the interspike period in inseminated spiking oocytes ( $n=12$ )



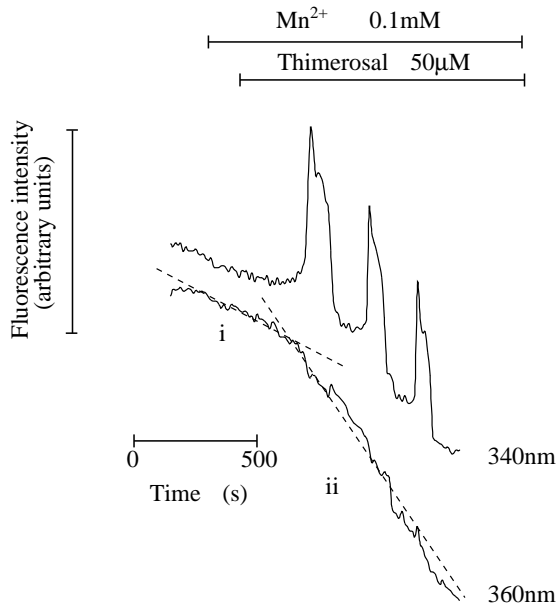
**Fig. 1.** Patterns of manganese ( $Mn^{2+}$ )-quench associated with the first fertilisation induced  $Ca^{2+}$  spike. The 360 nm trace shows quenching of fura-2 due to  $Mn^{2+}$  entry. The 340 nm trace indicates changes in cytosolic  $Ca^{2+}$ .  $Mn^{2+}$  (0.1 mM) was added as indicated by the bar. Typical response showing the basal quench rate in the latent period leading up to the first spike (i), the stimulated quench rate associated with the first spike (ii) and the post-spike quench rate (iii).

was, on average, 1.6-fold greater than that measured in oocytes imaged in the same optical field that had not responded to insemination ( $n=13$ ;  $P=0.0001$ ). In a duplicate experiment, the average ratio was 1.5 ( $n=7$ ,  $P=0.037$ ). Since the oocytes that did not spike after insemination may be considered abnormal, we also compared the quench rate in the interspike period in fertilised spiking oocytes with that in uninseminated (control) oocytes imaged in the same optical field. The quench rate measured in the interspike period of inseminated spiking oocytes ( $n=8$ ), was on average, 1.6-fold greater than that measured in control non-spiking oocytes ( $n=5$ ;  $P=0.026$ ). In a duplicate experiment, the average ratio was 1.4 ( $n=5$ ;  $P=0.04$ ). Thus, not only is there a stimulated rate of fluorescence quenching associated with the early spikes in most oocytes, but there is also a stimulated, but smaller rate of quenching in the interspike period.

The fact that the period between  $Ca^{2+}$  spikes in the fertilisation train is relatively long, combined with the marked stimulation of  $Mn^{2+}$  entry associated with the first spike, meant that, in most oocytes, the fura-2 in the oocyte was completely quenched before the second or third spike occurred. In order to see if there was also a stimulated rate of fluorescence quenching associated with later spikes, oocytes were inseminated and allowed to spike for intermediate (30–40 minutes) or long (1 hour 40 minutes) time periods postinsemination before  $Mn^{2+}$  was added. In the former case, there was a stimulated quench rate associated with later spikes in 14/16 oocytes (Fig. 2). When studying the longer periods, it was not possible to monitor the whole train because fura-2 was bleached during the course of the experiments. Therefore fura-2 was added to oocytes that had been fertilised 1 hour earlier, so that  $Mn^{2+}$  entry could be monitored at these later times. There was a small stimulation of fluorescence quench associated with the spike



**Fig. 2.** Pattern of manganese ( $Mn^{2+}$ ) quench associated with later spikes. Spermatozoa were added at arrow. Oocytes were allowed to spike for  $\approx 40$  minutes postinsemination before adding  $Mn^{2+}$  (bar). Fluorescence quenching due to  $Mn^{2+}$  entry was monitored at 360 nm, changes in cytosolic  $Ca^{2+}$  at 340 nm. The quench rate in the interspike period (i) and the stimulated quench rate associated with the third spike (ii) are indicated.



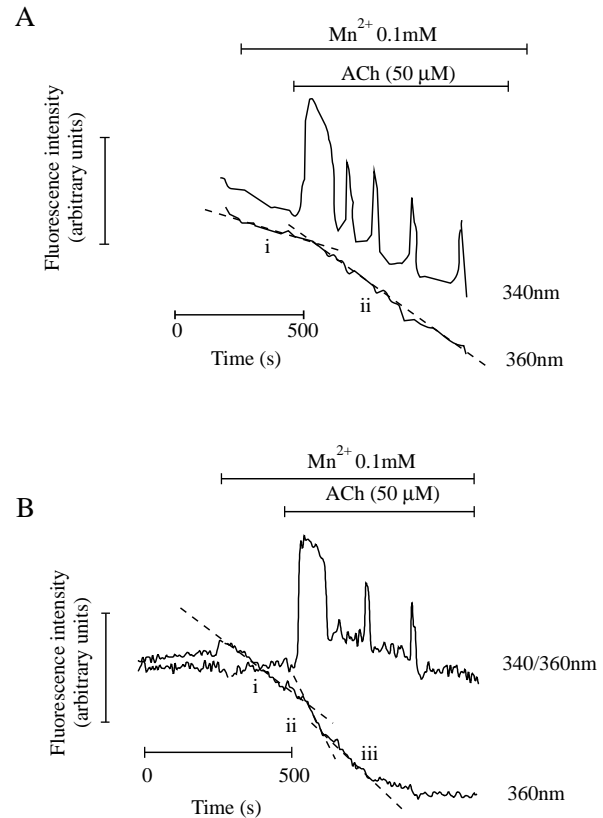
**Fig. 3.** Pattern of manganese ( $Mn^{2+}$ ) quench associated with the train of  $Ca^{2+}$  spikes induced by  $50 \mu M$  thimerosal. Fluorescence quenching due to  $Mn^{2+}$  entry was monitored at  $360 \text{ nm}$ , changes in cytosolic  $Ca^{2+}$  at  $340 \text{ nm}$ . The basal rate of quench in the unstimulated oocyte and in the latent period leading up to the first spike (i) and the stimulated quench rate associated with the first and subsequent spikes (ii), are indicated.

generated late in the fertilisation train (1 hour 40 minutes postinsemination; results not shown).

#### Increased divalent cation influx associated with spiking in response to thimerosal and acetylcholine

A train of  $Ca^{2+}$  spikes like that seen at fertilisation can be induced by the oxidising reagent thimerosal. These spikes are also reliant on extracellular  $Ca^{2+}$  and stop abruptly when  $Ca^{2+}$  is removed from the extracellular bathing medium. Spiking resumes immediately on reintroduction of  $Ca^{2+}$  to the extracellular bathing medium (unpublished result). Fig. 3 shows that, although there is a stimulated rate of fluorescence quenching associated with the train of spikes induced by thimerosal which commences with the onset of the first spike, there was little evidence for a reduction in the quench rate during the interval between spikes. Note that the spiking induced by this concentration of thimerosal ( $50 \mu M$ ) occurs at a much higher frequency than that seen at fertilisation.

Stimulation with acetylcholine induces spikes of a shorter duration and of a higher frequency than that seen at fertilisation. Fig. 4A illustrates that the pattern of cation influx associated with these spikes resembles that associated with thimerosal-induced  $Ca^{2+}$  spikes. There is a constant stimulation in the rate of fluorescence quenching which coincides with the onset of the first spike. Again, as seen with thimerosal, the frequency of spiking is much higher than that seen at fertilisation. The frequency of spiking induced by this concentration of acetylcholine varied and, in one instance, where the frequency of spiking was quite low, a stimulation of cation influx could be seen associated with each spike (Fig. 4B).



**Fig. 4.** Patterns of manganese ( $Mn^{2+}$ ) quench associated with a train of  $Ca^{2+}$  spikes induced by  $50 \mu M$  acetylcholine (ACh). Fluorescence quenching due to  $Mn^{2+}$  entry was monitored at  $360 \text{ nm}$ , changes in cytosolic  $Ca^{2+}$  using the  $340 \text{ nm}$  ratio.  $Mn^{2+}$  ( $0.1 \text{ mM}$ ) was added as indicated by the bar. (A) The basal rate of quench in the unstimulated oocyte and in the latent period leading up to the first spike (i) and the stimulated quench rate associated with the first and subsequent spikes (ii), in an oocyte spiking at high frequency are indicated; (B) Response of an oocyte spiking at a lower frequency showing the basal quench rate in the latent period leading up to the first spike (i), the stimulated quench rate associated with the first spike (ii) and the post-spike quench rate (iii).

#### A capacitative $Ca^{2+}$ entry pathway exists in mouse oocytes

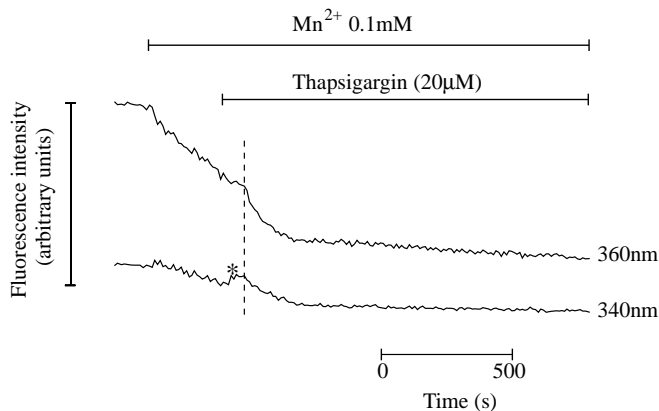
In many cell types,  $Ca^{2+}$  entry is triggered by the depletion of intracellular stores, a mechanism known as 'capacitative'  $Ca^{2+}$  entry. Capacitative  $Ca^{2+}$  entry can be revealed using the specific microsomal pump inhibitor, thapsigargin, to drain the intracellular stores of their  $Ca^{2+}$  content. Exposure of unfertilised oocytes to  $20 \mu M$  thapsigargin caused a transient rise in the  $[Ca^{2+}]_i$  (\*, Fig. 5). This rise in  $[Ca^{2+}]_i$  was associated with a stimulated rate of fluorescence quenching (Fig. 5). In all instances, the stimulated rate of fluorescence quenching occurred after peak  $[Ca^{2+}]_i$  was reached. This stimulated  $Mn^{2+}$  entry could be due either to a decline in the luminal  $[Ca^{2+}]$  in the intracellular stores triggering capacitative  $Ca^{2+}$  entry, or to the transient rise in  $[Ca^{2+}]_i$ . Several experiments were carried out to study the effect of store depletion on cation influx once the cytosolic  $[Ca^{2+}]$  had returned to near resting levels.

In a quiescent oocyte, under normal conditions, the removal and readdition of external  $Ca^{2+}$  had very little effect on the

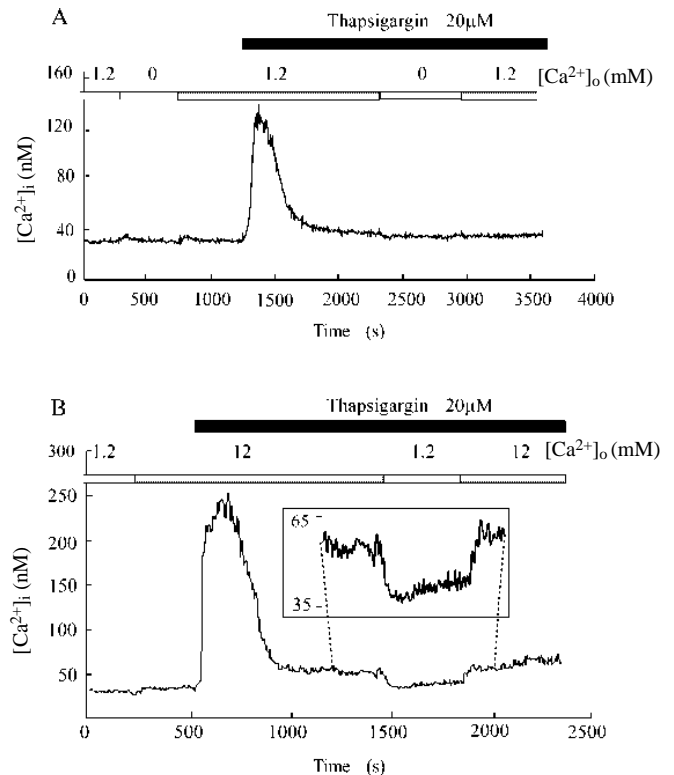
resting cytosolic  $\text{Ca}^{2+}$  level (Fig. 6A). Addition of 20  $\mu\text{M}$  thapsigargin in the presence of 1.2 mM external  $\text{Ca}^{2+}$  caused a transient rise in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  release from intracellular stores (Fig. 6A). These stores cannot reaccumulate  $\text{Ca}^{2+}$  due to pump inhibition by thapsigargin.  $[\text{Ca}^{2+}]_i$  did not return to its original resting level but remained slightly elevated perhaps due to the activation of a sustained capacitative  $\text{Ca}^{2+}$  entry pathway (Fig. 6A). The removal and reintroduction of  $[\text{Ca}^{2+}]_o$  resulted in very slight reductions and increases in  $[\text{Ca}^{2+}]_i$  respectively. It appeared then, that if a capacitative entry component existed, it was very small.

In an attempt to reveal the presumptive capacitative  $\text{Ca}^{2+}$  entry component more convincingly, oocytes were exposed to a higher  $[\text{Ca}^{2+}]_o$  (12 mM). Raising  $[\text{Ca}^{2+}]_o$  from 1.2 mM to 12 mM caused a slight elevation in the resting  $\text{Ca}^{2+}$  level in a quiescent egg with full stores (Fig. 6B). Addition of 20  $\mu\text{M}$  thapsigargin, caused a transient rise in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  release from intracellular stores. In the presence of 12 mM  $[\text{Ca}^{2+}]_o$ ,  $[\text{Ca}^{2+}]_i$  clearly remained elevated, presumably due to the activation of a capacitative  $\text{Ca}^{2+}$  entry pathway (Fig. 6B). On reduction of  $[\text{Ca}^{2+}]_o$  to 1.2 mM, resting  $\text{Ca}^{2+}$  levels were re-established (Fig. 6B). Restoring  $[\text{Ca}^{2+}]_o$  to 12 mM again caused a sustained rise in  $[\text{Ca}^{2+}]_i$  due to stimulated  $\text{Ca}^{2+}$  entry. The capacitative  $\text{Ca}^{2+}$  entry component is clearly rather small because high  $\text{Ca}^{2+}$  concentrations are needed to reveal it.

The  $\text{Ca}^{2+}$  influx triggered after depletion of intracellular  $\text{Ca}^{2+}$  stores with thapsigargin was also followed by monitoring the quench of fluorescence due to  $\text{Mn}^{2+}$  entry. Oocytes were incubated in either H6 buffer alone or in H6 buffer containing 20  $\mu\text{M}$  thapsigargin (the effects of which are irreversible) for 15 minutes to deplete intracellular  $\text{Ca}^{2+}$  stores. Both sets of eggs were then washed into nominally  $\text{Ca}^{2+}$ -free medium to ensure the intracellular stores remained empty and loaded with fura-2.  $\text{Mn}^{2+}$  was then introduced and the rate of fluorescence quenching monitored. In two out of three experiments, there was no difference between the control and the thapsigargin-treated oocytes. In the remaining experiment, the rate of fluorescence quenching in the thapsigargin-treated oocytes ( $n=6$ ) was 1.4-fold greater than that measured in



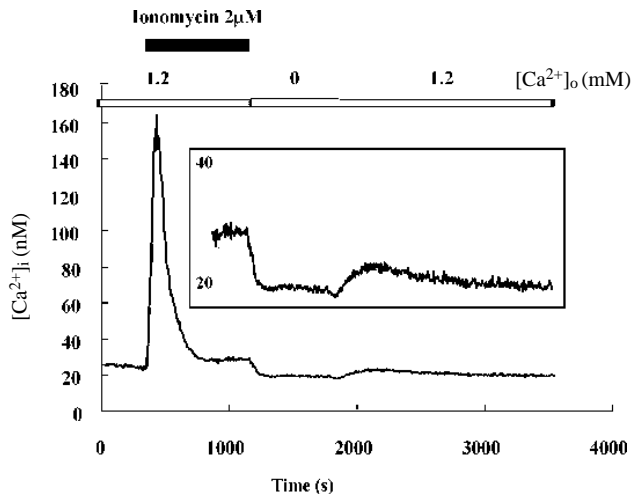
**Fig. 5.** Thapsigargin causes a transient release of  $\text{Ca}^{2+}$  from intracellular stores. (\*, 340 nm trace) which is associated with a stimulated rate of manganese ( $\text{Mn}^{2+}$ ) quench (360 nm trace). The dotted line indicates that the stimulated rate of fluorescence quenching occurs after peak  $[\text{Ca}^{2+}]_i$  has been reached.



**Fig. 6.** High concentrations of  $\text{Ca}^{2+}$  are needed to reveal 'capacitative' or store depletion induced  $\text{Ca}^{2+}$  entry. Traces show changes in cytosolic  $\text{Ca}^{2+}$ . (A) Extracellular  $\text{Ca}^{2+}$  was alternated between 1.2 mM (hatched bar) and nominally  $\text{Ca}^{2+}$  free (open bar) as indicated, before and after addition of thapsigargin (solid bar). (B) Extracellular  $\text{Ca}^{2+}$  was alternated between 1.2 mM (open bar) and 12 mM (hatched bar), before and after addition of thapsigargin (solid bar). The inset shows changes at greater resolution. The difference in amplitude between Fig. 6A and Fig. 6B reflects the usual range of variability between individual oocytes rather than an increase in  $\text{Ca}^{2+}$  entry caused by increasing  $[\text{Ca}^{2+}]_o$  to 12 mM.

control oocytes ( $n=6$ ;  $P=0.0025$ ). In this particular experiment, the oocytes had extruded their polar bodies. In contrast, no polar body extrusion was seen in the other two experiments, perhaps indicating that, in these cases, treatment with thapsigargin had caused a less extensive depletion of stores, as the extrusion of polar bodies is dependent on the amplitude of the  $\text{Ca}^{2+}$  transient (Vincent et al., 1992).

Ionomycin has also been reported to enhance  $\text{Ca}^{2+}$  influx by stimulating capacitative  $\text{Ca}^{2+}$  entry (Morgan and Jacob, 1994). These investigators showed that at concentrations  $\leq 1 \mu\text{M}$  this is the ionophore's primary action. Removal and readdition of only 1.2 mM  $[\text{Ca}^{2+}]_o$  revealed a capacitative entry component after depleting the internal stores with 1  $\mu\text{M}$  ionomycin (Fig. 7). Note that when 1.2 mM  $[\text{Ca}^{2+}]_o$  was reapplied, the  $[\text{Ca}^{2+}]_i$  did not return to the previous plateau level but gradually declined to the original resting level probably resulting from the washout of ionomycin, the effects of which are reversible (Fig. 7) (Morgan and Jacob, 1994). If ionomycin were still translocating  $\text{Ca}^{2+}$  across the membrane after this period of time  $[\text{Ca}^{2+}]_i$  would remain elevated. Thus, under these circumstances,  $\text{Ca}^{2+}$ -influx fills up the intracellular stores, over a



**Fig. 7.** Ionomycin reveals 'capacitative'  $\text{Ca}^{2+}$  entry induced by store depletion. Traces show changes in cytosolic  $\text{Ca}^{2+}$ . Extracellular  $\text{Ca}^{2+}$  was alternated between 1.2 mM (hatched bar) and nominally  $\text{Ca}^{2+}$  free (open bar) as indicated. Ionomycin was present only for the period indicated (solid bar).

period of 15 to 20 minutes, eventually shutting down capacitative  $\text{Ca}^{2+}$  entry. Enhancement of cation influx by ionomycin was also monitored using the  $\text{Mn}^{2+}$ -quench technique. Stores were depleted by treating oocytes with 1 or 2  $\mu\text{M}$  ionomycin in nominally  $\text{Ca}^{2+}$ -free medium. The ionomycin was washed out and the oocytes were loaded with fura-2 for 30 minutes. As indicated by the result in Fig. 7, this should completely remove the ionomycin.  $\text{Mn}^{2+}$  was then introduced and the quench in fluorescence monitored. In both experiments, the quench rate in the ionomycin-treated oocytes was significantly greater than that in control oocytes imaged in the same optical field. The quench rate in oocytes treated with 1  $\mu\text{M}$  ionomycin was 1.35 times that in control oocytes ( $n \geq 12$ ,  $P = 0.043$ ) while the quench rate in oocytes treated with 2  $\mu\text{M}$  ionomycin was 1.5 times that in control oocytes ( $n \geq 8$ ,  $P = 0.0001$ ). Morgan and Jacob (1994) have previously observed a dose-dependent effect of ionomycin in human umbilical vein endothelial cells. Clearly a capacitative influx pathway exists and this could account for both the stimulated cation entry associated with the spike and that which occurs in the interspike period.

## DISCUSSION

Previous studies have suggested that an increased rate of  $\text{Ca}^{2+}$  influx is a likely consequence of oocyte fertilisation (Igusa and Miyazaki, 1983; Kline and Kline 1992a; Cheek et al., 1993). In this study, we have demonstrated directly using  $\text{Mn}^{2+}$  as a  $\text{Ca}^{2+}$  surrogate that there is a stimulation of cation entry associated with the onset of the first and subsequent fertilisation  $\text{Ca}^{2+}$  spikes. Cation influx slows down following the downstroke of each spike but usually remains higher than the basal rate of influx measured in the latent period before the first spike. No significant increase in  $\text{Ca}^{2+}$  influx was observed during the latent period before the first  $\text{Ca}^{2+}$  spike occurs. While it is possible that the sperm precipitates the train of  $\text{Ca}^{2+}$

spikes by causing an influx of  $\text{Ca}^{2+}$ , which saturates the intracellular  $\text{Ca}^{2+}$  stores, the resolution of the  $\text{Mn}^{2+}$ -quench technique may not have been sensitive enough to detect such a very small increase. The  $\text{Ca}^{2+}$  influx mechanism operating at fertilisation is clearly linked to periodic  $\text{Ca}^{2+}$  spiking and may be potentiated when  $\text{Ca}^{2+}$  is released from the intracellular  $\text{Ca}^{2+}$  stores as occurs during a  $\text{Ca}^{2+}$  spike. We found that spike-associated entry is not as apparent in mouse oocytes stimulated to spike repetitively by thimerosal or acetylcholine application. Instead, in response to these stimuli, there is a continuous increase in cation influx underlying  $\text{Ca}^{2+}$  spiking, which initiates with the onset of the first spike. Previous examinations of the patterns of  $\text{Ca}^{2+}$  entry underlying  $\text{Ca}^{2+}$  spiking using the  $\text{Mn}^{2+}$ -quench technique in other cell types have produced differing results. A spike associated entry component was observed on hormonal stimulation of AR42J cells (Loessberg et al., 1991) and during spontaneous  $\text{Ca}^{2+}$  spiking in MDCK-F cells (Wojnowski et al., 1994). In contrast, the  $\text{Ca}^{2+}$  influx was not spike-associated in either endothelial cells (Jacob, 1990) or HeLa cells (Thorn, 1995).

It has been shown, in mouse oocytes, that current through voltage-operated  $\text{Ca}^{2+}$  channels (VOCs) is enhanced immediately following fertilisation (Yamashita, 1982). VOCs are known to conduct  $\text{Mn}^{2+}$  (Shibuya and Douglas, 1992) and such channels may be responsible for the spike-associated influx seen in AR42J cells which are electrically excitable (Kusano and Gainer, 1991).  $\text{Ca}^{2+}$  spiking in the hamster oocyte is accompanied by periodic hyperpolarising responses due to the activation of a  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  conductance (Miyazaki and Igusa, 1982). It is conceivable that a  $t$ -type  $\text{Ca}^{2+}$  channel could be activated on the depolarising phase of this hyperpolarisation and contribute to  $\text{Ca}^{2+}$  influx. The presence of a  $t$ -type  $\text{Ca}^{2+}$  channel in fertilised mouse oocytes has been demonstrated (Day et al., 1995). However, in mouse oocytes, there is only a very small and often undetectable hyperpolarisation associated with each fertilisation  $\text{Ca}^{2+}$  spike (Igusa et al., 1983). In addition, pimozone, which inhibits the  $t$ -type  $\text{Ca}^{2+}$  channel in fertilised mouse oocytes (Dr Margot Day; unpublished results) had no effect on the fertilisation  $\text{Ca}^{2+}$  spikes. Thus, it is unlikely that such a mechanism is involved in mediating the spike-associated  $\text{Ca}^{2+}$  influx in mouse oocytes.

Depletion of the intracellular  $\text{Ca}^{2+}$  stores in mouse oocytes with thapsigargin and more extensive depletion of stores with ionomycin causes a transient rise in  $[\text{Ca}^{2+}]_i$  and stimulates a divalent cation influx across the oolemma after peak  $[\text{Ca}^{2+}]_i$  is reached, indicating that a capacitative  $\text{Ca}^{2+}$  entry mechanism exists in these cells. The term 'capacitative'  $\text{Ca}^{2+}$  entry was coined originally by Putney (1986, 1990) to introduce the idea that the influx of external  $\text{Ca}^{2+}$  is regulated by the state of fullness of the intracellular  $\text{Ca}^{2+}$  stores. The microsomal pump inhibitor thapsigargin is used routinely to reveal capacitative  $\text{Ca}^{2+}$  entry in many cell types. However, thapsigargin is not very effective at emptying the intracellular  $\text{Ca}^{2+}$  stores in mouse oocytes (Kline and Kline, 1992b). A concentration of the drug over an order of magnitude greater than that routinely used in other cell types is needed to deplete the stores appreciably. Even then, subsequent application of the  $\text{Ca}^{2+}$  ionophore A23187 still releases a significant amount of  $\text{Ca}^{2+}$  from the stores (Kline and Kline, 1992b). It may be that the microsomal  $\text{Ca}^{2+}$  pumps in mature mouse oocytes are less sensitive to the drug or that the pumps are not very active such

that their inhibition does not result in a significant change in the content of the intracellular  $\text{Ca}^{2+}$  stores. Ionomycin is much more effective at emptying the intracellular  $\text{Ca}^{2+}$  stores in mouse oocytes. The concentrations used in this study are comparable to those used by Morgan and Jacob (1994) on endothelial cells. The effects of ionomycin, unlike those of thapsigargin, are reversible. Thus, on reintroduction of  $[\text{Ca}^{2+}]_o$  after exposure of oocytes to ionomycin in  $\text{Ca}^{2+}$ -free medium, the  $\text{Ca}^{2+}$  stores eventually refill, shutting off capacitative  $\text{Ca}^{2+}$  entry (Fig. 7).

The nature of the signalling pathway that conveys the depleted state of the intracellular  $\text{Ca}^{2+}$  stores in cells to the  $\text{Ca}^{2+}$ -influx channels on the plasma membrane is unknown. One hypothesis is that on store depletion a soluble calcium influx factor (CIF) diffuses from the empty store to  $\text{Ca}^{2+}$ -influx channels on the plasma membrane and activates them (Randriamampita and Tsien, 1993). Another proposal is that sensitisation of  $\text{InsP}_3$  receptors on intracellular stores causes their conformation to change. This in turn allows them to couple with  $\text{Ca}^{2+}$ -influx channels on the plasma membrane and activate  $\text{Ca}^{2+}$  entry (Irvine, 1990; Berridge, 1990 and Penner et al., 1993 for a review). In either case, it is most likely to be peripheral  $\text{Ca}^{2+}$  stores that communicate with the  $\text{Ca}^{2+}$  influx channels on the oolemma. There is evidence that a proportion of the  $\text{InsP}_3$  receptors are localised on a specialised region of the endoplasmic reticulum that is closely associated with the plasma membrane (Lievremont et al., 1994). In addition, these peripheral stores will take the longest time to refill following spike recovery because they are located where the concentration of  $\text{InsP}_3$  is at its highest, at its site of production near the membrane. During a train of  $\text{Ca}^{2+}$  spikes, some peripheral stores may never have a chance to refill so that they gate continuous  $\text{Ca}^{2+}$  influx to stores deeper within the cytosol.

The main conclusion from these studies is that capacitative  $\text{Ca}^{2+}$  entry, through subolemmal  $\text{Ca}^{2+}$  stores, is triggered during the first fertilisation spike. The decrease in  $\text{Ca}^{2+}$  entry following the recovery of the spike probably indicates that most of the peripheral stores mediating  $\text{Ca}^{2+}$  entry have begun to refill, thus attenuating capacitative  $\text{Ca}^{2+}$  entry. The interspike entry that persists takes a longer time to saturate the deeper cytoplasmic stores until they again become sensitive to regenerative  $\text{Ca}^{2+}$  release. This is reflected in the very long interspike interval or low frequency of spiking seen in response to fertilisation. In contrast to this situation,  $\text{Ca}^{2+}$  entry induced by acetylcholine or thimerosal does not decrease in the interspike period. In the presence of these agents, and perhaps because they stimulate  $\text{InsP}_3$  receptors localised on the subolemmal  $\text{Ca}^{2+}$  stores more effectively than occurs after fertilisation, these peripheral stores must remain sufficiently depleted to maintain a constant rate of  $\text{Ca}^{2+}$  entry. This causes a more rapid saturation of the deeper cytoplasmic stores, reducing the interspike interval leading up to the pacemaker rise in  $\text{Ca}^{2+}$  and regenerative  $\text{Ca}^{2+}$  release and, as observed, a comparatively higher frequency of spiking.

Thus continuous  $\text{Ca}^{2+}$  entry underlies  $\text{Ca}^{2+}$  spiking at fertilisation. This entry may be linked to periodic  $\text{Ca}^{2+}$  release by a capacitative mechanism. As the stores empty on the first fertilisation spike, capacitative  $\text{Ca}^{2+}$  entry is triggered. This entry may contribute to the magnitude and duration of the spike. During the recovery of each spike, most  $\text{Ca}^{2+}$  stores begin to refill thus attenuating capacitative  $\text{Ca}^{2+}$  entry. We propose that

the small degree of entry that persists is through peripheral stores which take the longest time to refill because they are located where the concentration of  $\text{InsP}_3$  is at its highest, at its site of production near the membrane. This continuous influx of  $\text{Ca}^{2+}$  sets the frequency of spiking by determining the time taken to refill the internal stores to a point where they are again sensitive enough to initiate the next spike.

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