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Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse

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SUMMARY

Fusion of sperm and egg plasma membranes is an early and essential event at fertilization but it is not known if it plays a part in the signal transduction mechanism that leads to the oscillations in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that accompany mammalian egg activation. We have used two independent fluorescence methods and confocal microscopy to show that cytoplasmic continuity of egg and sperm precedes the onset of the first $[Ca^{2+}]_i$ increase in mouse eggs. The Ca^{2+} indicator dye Ca^{2+}-green dextran was microinjected and its transfer from egg to sperm was monitored. We found that it occurred before, and without a requirement for, any detectable $[Ca^{2+}]_i$ increase in the egg. In separate experiments $[Ca^{2+}]_i$ changes were recorded in populations of eggs, using fura red, and the eggs fixed at various times after some of the eggs had shown a $[Ca^{2+}]_i$ transient. Fusion of the sperm and egg was then assessed by Hoechst dye transfer. All eggs that showed a $[Ca^{2+}]_i$ increase had a fused sperm but more than half of the eggs contained a sperm but had not undergone a $[Ca^{2+}]_i$ increase. These data indicate that sperm-egg fusion precedes $[Ca^{2+}]_i$ changes and we estimate that the elapsed time between sperm-egg fusion and the onset of the $[Ca^{2+}]_i$ oscillations is 1-3 minutes. Finally, sperm-egg fusion was prevented by using low pH medium which reversibly prevented $[Ca^{2+}]_i$ oscillations in eggs that had been inseminated. This was not due to disruption of signalling mechanisms, since $[Ca^{2+}]_i$ changes still occurred if low pH was applied after the onset of oscillations at fertilization. $[Ca^{2+}]_i$ changes also occurred in eggs in low pH in response to the muscarinic agonist carbachol. These data are consistent with the idea that the $[Ca^{2+}]_i$ signals that occur in mammalian eggs at fertilization are initated by events that are closely coupled to the fusion of the sperm and egg membranes.

Key words: calcium, fertilization, sperm-egg fusion, mouse

INTRODUCTION

The fertilizing sperm activates all vertebrate and invertebrate eggs by generating $[Ca^{2+}]_i$ transients in the egg cytoplasm (Jaffe, 1983, Whitaker and Steinhardt, 1982; Nuccitelli, 1991; Swann and Ozil, 1994). In nearly all cases the $[Ca^{2+}]_i$ transients are generated by a signal transduction mechanism that leads to Ca^{2+} release from intracellular stores. However, it is unclear which of the many possible signal transduction pathways provides the initial trigger for this Ca^{2+} release (Nuccitelli, 1991; Miyazaki et al., 1993; Whitaker and Swann, 1993). There are two classes of hypotheses that could explain signal transduction at fertilization. One class involves cell messenger-linked transmembrane receptors: sperm may bind to a receptor, generating inositol 1,4,5-triphosphate ($InsP_3$) *via* a G-protein pathway (Foltz and Schilling, 1993; Miyazaki et al., 1993; Schultz and Kopf, 1995) or perhaps via a tyrosine kinase pathway (Foltz and Shilling, 1993; Schultz and Kopf, 1995). The other class of hypotheses is based on the idea that an activating diffusible messenger is introduced into the egg by the sperm when the sperm fuses with the egg and cytoplasmic continuity is established (Dale et al., 1985; Whitaker et al., 1989; Swann, 1993). The identity of such a diffusible messenger is not generally known. $InsP_3$ (Iwasa et al., 1990; Tosti et al., 1993), cyclic GMP (Whalley et al., 1992) and Ca^{2+} itself (Jaffe, 1991) have all been proposed as candidates in invertebrate eggs. In mammalian eggs, it has been suggested that the activating messenger may be a novel protein called oscillin (Parrington et al., 1996).

The hypothesis of a diffusible messenger requires that cytoplasmic continuity between gametes obtains before the initial $[Ca^{2+}]_i$ transient occurs. The timing of sperm-egg fusion in the sea urchin has been determined with some precision using electrophysiological (McCulloh and Chambers, 1992), ultrastructural (Longo et al., 1986) and dye-transfer (Hinkley et al., 1986) methods. It occurs 10-15 seconds before a $[Ca^{2+}]_i$ transient is generated. This time delay that elapses between fusion and activation is known as the latent period (Allen and Griffen, 1958; Whitaker and Swann, 1993). However, despite early reports on an activating sperm factor (Dale et al., 1985), no real progress has been made in identifying the diffusible messenger in sea urchins. Mammalian sperm, in contrast, contain a protein that mimics the fertilizing sperm in generating a repetitive series of $[Ca^{2+}]_i$ oscillations when injected into unfertilized mouse, hamster and human eggs (Swann, 1990, 1994; Homa and Swann, 1994; Parrington et al., 1995). But

there are no data that define the timing of sperm-egg fusion in mammalian eggs, so it is not clear whether the sperm factor would have the early opportunity of diffusing from sperm to egg to generate the activating $[Ca^{2+}]_i$ transient. It is also not clear whether sperm-egg membrane fusion is required for the generation of $[Ca^{2+}]_i$ changes in mammalian or sea urchin eggs.

We used two methods to look at the timing of sperm-egg fusion in mouse eggs. In the first, the diffusion of a fluorescent dye, Ca^{2+}-green dextran, from egg to sperm during fertilization was examined using a confocal microscope. The same dye was also used to monitor $[Ca^{2+}]_i$. We found that the dye could be detected in the sperm before we detected a $[Ca^{2+}]_i$ transient in the egg. The second method took advantage of the fact that we can measure $[Ca^{2+}]_i$ in individual identified eggs within a population, using the Ca^{2+} indicator dye fura red. The eggs were also pre-labelled with Hoechst 33342 (Hinkley et al., 1986). When one or two of the eggs in a field of fertilizing eggs underwent $[Ca^{2+}]_i$ increases, we fixed the population. We found Hoechst 33342 dye transfer to sperm in eggs that had not undergone $[Ca^{2+}]_i$ transients. Both methods demonstrated that cytoplasmic continuity is established between mouse egg and sperm several minutes before the first $[Ca^{2+}]_i$ increase occurs. We were able to estimate a mean latent period in mouse eggs of around 1-3 minutes. We have also examined if membrane fusion is required for $[Ca^{2+}]_i$ oscillations by preventing fusion with low pH (Yanagimachi et al., 1980). Low pH prevented the initation of $[Ca^{2+}]_i$ oscillations after sperm addition to eggs in a reversible manner. These data suggest that gamete fusion is a prerequisite for $[Ca^{2+}]_i$ signalling and egg activation at fertilization in the mouse.

MATERIALS AND METHODS

Preparation of gametes

MF1 females were superovulated by intraperitoneal injection of 5-10 i.u. pregnant mare's serum gonadotrophin (PMSG) followed by 5-10 i.u. human chorionic gonadotrophin (HCG) 48 hours apart. Oviducts were taken from animals killed by cervical dislocation 13-15 hours post-HCG. The eggs were released into M2 medium containing 0.3 mg/ml hyaluronidase to remove cumulus cells and maintained in M2 supplemented with 4 mg/ml bovine serum albumin, BSA (ICN), at 37°C. The composition of M2 medium is given by Fulton and Whittingham (1978). The zona pellucida was removed from eggs preloaded with fluorescent dye by brief treatment with acid tyrode solution (pH 2.5). Eggs were then lightly attached to polylysine coated coverslips that formed the base of a chamber containing M2 medium. In some experiments eggs were preloaded with dyes before attachment to polylysine coated coverslips. Sperm were collected from the cauda epididymis of CBA male mice of proven fertility that were culled by cervical dislocation. Sperm were released into T6 medium plus 15 mg/ml BSA (fraction V) which was incubated for approx 2 hours at 37°C, 5% CO_2 before adding to the drops containing the eggs (Lee et al., 1988). Unless stated otherwise, media and reagents such as carbochol were from Sigma (UK).

Confocal microscopy of Ca^{2+}-green dextran loaded eggs

The fluorescence of the Ca^{2+}-sensitive dye Ca^{2+}-green dextran (10,000 M_r; Molecular Probes, Eugene, OR) was measured using a Leica CLSM (Leica Lasertechnik, Heidelberg) with a Kr/Ar laser, 488 nm excitation and a 520 nm longpass emission filter. Calibrating the single wavelength dye in terms of Ca^{2+} concentration is not straightforward and was not attempted here, since it was the timing rather than the magnitude of the $[Ca^{2+}]_i$ transients that interested us here. The dye was microinjected into unfertilized mouse eggs using pressure pulses (Swann, 1990). The injection solutions consisted of Ca^{2+} green dextran with or without BAPTA (1,2-(bis(2-aminophoxy)ethane-N,N,N′,N′-tetraacetic acid) in an injection buffer; 120 mM KCl, 20 mM Hepes, pH 7.5. After insemination of the eggs at a sperm density of approx. 10^5 sperm/ml, we scanned the egg at regular intervals and manually adjusted the z-section, looking for dye signals associated with $[Ca^{2+}]_i$ increases and with the sperm. Acquired images were stored on magneto-optical disks.

Measurement of $[Ca^{2+}]_i$ with fura red and gamete fusion with H33342

In experiments where H33342 dye transfer was to be monitored, the eggs were loaded with fura red (Molecular Probes) and Hoechst 33342 dyes by a 30 minute incubation in 1 μg/ml H33342, followed by a 10 minute incubation in 2 μM Fura red-acetoxymethylester (fura red-AM) plus 0.005% pluronic. H33342 and fura red-AM were dissolved in M2 medium from stocks (1 mg/ml and 2 mM respectively) made up in dimethyl sulfoxide. The chamber containing the eggs, that had adhered to the base, was placed on the stage of a Nikon Diaphot microscope illuminated with epifluorescence via a 20×, 0.75 NA lens. The eggs were inseminated with capacitated sperm and the fluorescence of fura red continuously monitored. The eggs were fixed, at different time points after insemination, by rapid (1-5 seconds) perfusion of the dish containing the eggs with 2% formaldehyde. The eggs were then examined for signs of sperm entry by illumination of the H33342 fluorescence (Hinkley et al., 1986; Conner and Gwatkin, 1988). The Ca^{2+} free medium used in some experiments consisted of M2 plus 5 mM EGTA (ethylene glycol-bis(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid). Fluorescence from fura red loaded eggs was measured using epifluorescene and a Newcastle Photometrics Multipoint System (Newcastle upon Tyne, UK). This system can monitor fluorescence

Fig. 1. Transfer of Ca^{2+}-green dextran dye from egg to sperm in BAPTA-injected eggs. Different parts of the sperm tail appear in different confocal sections, so the image is a sum of 5 sections. This has the effect of increasing the apparent signal in the egg relative to the sperm. The fluorescent dye spreads into the tail and the bulge represents a residual cytoplasmic droplet (not seen in all sperm). The inset shows the region of sperm entry at ×2 magnification. The egg is 70 μm in diameter.

from up to 16 eggs simultaneously using a CCD camera. In these experiments where H33342 dye transfer was also to be measured the fura red fluorescence was measured at a single wavelength by excitation at 490 nm; emission was measured with a 590 nm longpass filter. H33342 fluorescence was measured after fixation using 340-380 nm excitation and 470-490 nm emission filters.

Prevention of sperm egg membrane fusion

In most of these experiments the methods for measuring $[Ca^{2+}]_i$ and H33342 dye transfer were as described above. To prevent sperm-egg fusion, the eggs were placed in a bath on the microscope stage that was either at pH 6.1 or that contained 0.25% Tween 80 (as described in Chernomordik et al., 1993). After 15 minutes sperm were added to these eggs and then after 1 hour normal M2 medium was perfused onto the eggs. Many sperm that were firmly bound to eggs were not removed during this perfusion step. H33342 dye transfer was monitored after 1 hour in the test solutions (low pH medium or Tween 80 containing medium) and then at the end of the experiment. In some experiments H33342 dye transfer was not monitored and so eggs were only loaded with fura red and the fluorescence of fura red was monitored as the ratio of emission intensity with alternating 440 and 490 nm excitation wavelengths (Kurebayashi et al., 1993). This method was not used for eggs that had been loaded with H33342 since there was some spillover of fluorescence from H33342 at the 440 nm excitation.

Acrosome reaction assay

The acrosome reaction rates of mouse sperm were assessed by examining the lectin staining pattern using FITC labelled *Pisum sativum* agglutinin (Mendoza et al., 1992). Sperm were capaciated in T6 medium for 2 hours and then resuspended in M2 medium, M2 medium plus Tween 80, or pH 6.1 M2 medium. After a further 1 hour incubation in these media they were then fixed in 4% formaldehyde and then processed for lectin staining as described by Mendoza et al. (1992).

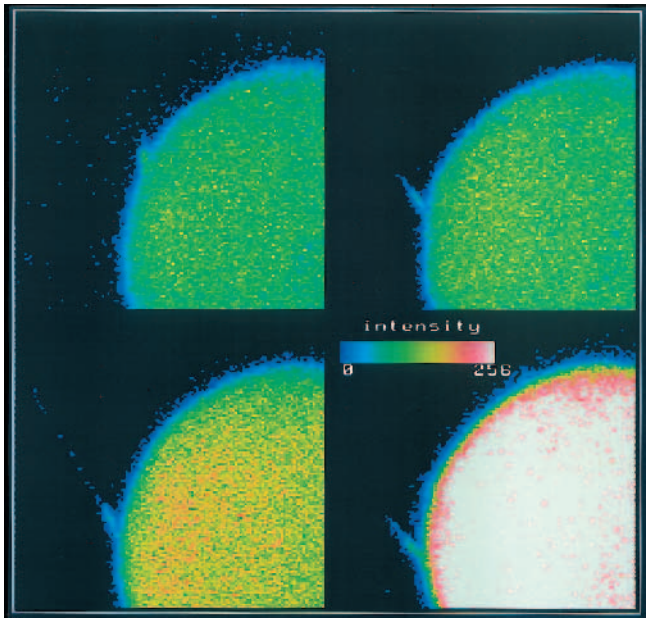
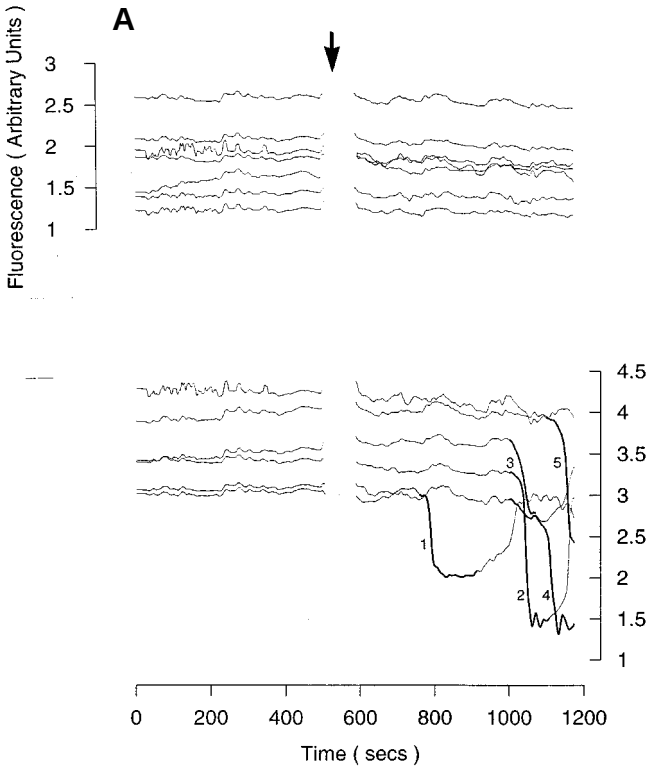


Fig 2. Transfer of Ca^{2+}-green dextran dye from egg to sperm at fertilization. Dye enters the sperm head and sperm tail before the onset of the $[Ca^{2+}]_i$ transient. The four images read left to right and top to bottom and were taken 6 seconds apart. The pseudocolour table indicates the relative fluorescence scale with green representing low $[Ca^{2+}]_i$ and white high $[Ca^{2+}]_i$.

RESULTS

Detection of sperm-egg fusion using Ca^{2+}-green dextran and confocal microscopy

We wanted to establish that we could detect diffusion of calcium-green dextran from the egg to the sperm, so we inseminated eggs that had been microinjected with a mixture of Ca^{2+}-green dextran and BAPTA, a Ca^{2+} chelator. Eggs were injected with approximately 1% volume of 10 mM BAPTA along with



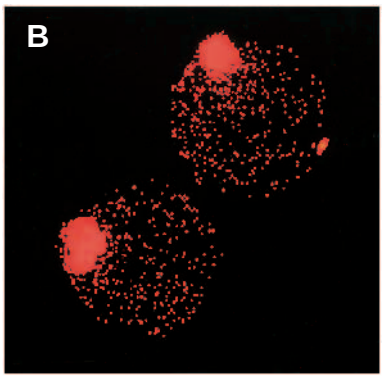


Fig 3. Correlation of $[Ca^{2+}]_i$ transients with Hoechst dye (H33342) uptake. (A) Fura red records showing $[Ca^{2+}]_i$ in each egg in a single field of eggs. Increases in $[Ca^{2+}]_i$ are seen as decreases in fura red fluorescence intensity. The five $[Ca^{2+}]_i$ transients are labelled in order of appearance and the rising phase of each $[Ca^{2+}]_i$ transient is shown in bold. The records end when fixative was added to the eggs, in this case at 1175 seconds. Records from 7 eggs that did not show a $[Ca^{2+}]_i$ increase in the same experiment are shown above for clarity. The arrow during the break in the traces indicates the time at which sperm were added to the drop containing the eggs. (B) An example of the H33342 images, showing two eggs, one of which has a H33342-labelled sperm associated with it. This sperm is the small fluorescent object in one of the eggs. Neither egg had undergone a $[Ca^{2+}]_i$ transient. The large fluorescent region within the egg is the egg DNA.

the Ca^{2+}-green dextran. BAPTA suppressed $[Ca^{2+}]_i$ transients completely in all of 7 eggs. Fig. 1 shows an example of this sort of experiment. A sperm can be seen as a fluorescent arc at the top of the egg. Calcium green dextran has diffused from the egg cytoplasm into the sperm head and mid-piece and has filled the cytoplasmic droplet present at the base of the mid-piece. By scanning in the z-axis is was possible to detect more than one Ca^{2+}-green loaded sperm in each of the BAPTA-injected egg; 3.6±0.39 (mean and s.e.m.) sperm per egg were detected. Since the dextran-coupled dye is completely membrane inpermeant, these observations establish that the 10,000 M_r dye diffuses into the sperm from the egg to detectable levels. They also demonstrate that sperm-egg fusion in mammals can occur in the absence of detectable increases in $[Ca^{2+}]_i$.

Detection of $[Ca^{2+}]_i$ transients and sperm-egg fusion in single eggs

In the next set of experiments we observed single eggs microinjected with Ca^{2+}-green dextran and then inseminated. All z planes in the egg were inspected by systematically moving the confocal section through the egg manually every 20-60 seconds. When a fluorescent sperm was seen, confocal images were generated every 3 or 6 seconds. In 6 cases, we detected a fluorescent sperm and 3.3±1.0 (mean and s.e.m) minutes later, we detected a $[Ca^{2+}]_i$ increase. An example of what we saw is shown in Fig. 2. During the initial stages of dye diffusion, when small amounts of Ca^{2+}-green dextran have diffused into the sperm, fluorescence is seen only in the sperm head. It is a limitation of the technique that the sperm can be seen only at an indeterminate time after cytoplasmic continuity is established. The longest interval that we measured between first detecting the sperm and the onset of the $[Ca^{2+}]_i$ transient was 7 minutes. We imagine that the shorter intervals we usually measured might be due to the time that elapsed between sperm-egg fusion and our detection of dye transfer. These observations demonstrate that continuity of sperm and egg cytoplasm can occur before the onset of the $[Ca^{2+}]_i$ transient in mouse eggs. A second method was used to estimate the interval between sperm-egg fusion and the onset of the $[Ca^{2+}]_i$ transient and to verify if fusion always occurs before a $[Ca^{2+}]_i$ increase.

Monitoring sperm-egg fusion and $[Ca^{2+}]_i$ transients in egg populations

We took advantage of a method introduced by Hinkley et al. (1986). The eggs are loaded with a vital DNA stain (Hoechst 33342) before fertilization. Eggs are then fixed at various times after fertilization. If sperm and egg have fused, the H33342 diffuses slowly into the sperm head and labels the sperm nucleus. Unfused sperm show no such dye transfer. A field of eggs that had been loaded with both H33342 and the Ca^{2+} indicator dye fura red were fertilized on the stage of a fluorescence imaging microscope. When one or several of the eggs had fired $[Ca^{2+}]_i$ transients (detected as a decrease in fura red fluorescence), the entire field was fixed (Fig. 3A). Within 10 minutes after fixation H33342 fluorescence was monitored to look for fused sperm. As expected, we found fused sperm in all eggs that had fired off a $[Ca^{2+}]_i$ transient. This shows that all eggs that had undergone a $[Ca^{2+}]_i$ increase have at least one fused sperm. As predicted from our experiments with Ca^{2+}-green transfer, we also found fused sperm (dye transfer) in eggs that had not fired a $[Ca^{2+}]_i$ transient (Fig. 3B), as well as unfertilized eggs that had neither a $[Ca^{2+}]_i$ transient, nor fused sperm. The relative frequencies in 6 experiments (83 eggs) are shown in Table 1. We invariably observed many sperm bound to the eggs after fixation in these experiments. Of these eggs it appears that some had undergone sperm-egg fusion, but had not yet fired a $[Ca^{2+}]_i$ transient at the time of fixation. This supports the hypothesis that fusion occurs before the onset of the $[Ca^{2+}]_i$ transient. We can also estimate the latency of the response from these data.

Table 1. Summary of results of experiments monitoring sperm fusion and $[Ca^{2+}]_i$ transients

	No $[Ca^{2+}]_i$ transient	$[Ca^{2+}]_i$ transient observed
No sperm fusion	50% (42/83)	0% (0/83)
One or more sperm fused*	30% (24/83)	20% (17/83)

*Four eggs were polyspermic. Of these, two had a $[Ca^{2+}]_i$ transient.

Estimating the latent period at fertilization in mouse eggs

If the latent period were very short, we should not have detected fused sperm prior to the $[Ca^{2+}]_i$ transient by either of the methods employed. In general, the shorter the time that elapses between sperm fusion and the $[Ca^{2+}]_i$ transient at a given fertilization rate, the less likely is the detection of sperm that have fused but not yet generated a $[Ca^{2+}]_i$ transient. The fertilization rate in these experiments can be estimated from the rate of occurrence of $[Ca^{2+}]_i$ transients. Although the time from insemination to the first $[Ca^{2+}]_i$ transient varied considerably, due to variations in the time it took for sperm to swim to the eggs, the rates were in the same range in all experiments. In three of six experiments, the rate was reasonably constant (Fig. 4). The mean rate was 0.018/seconds. On average in these three experiments we detected 1.3 (4/3) eggs with fused sperm that had not experienced a $[Ca^{2+}]_i$ transient. This suggests that the latent period (the time that elapses between sperm-egg fusion and the onset of the $[Ca^{2+}]_i$ transient) is around 75 (1.3/0.018) seconds. The largest error in this estimate is the counting error (sqrt(n)), giving an upper estimate of the mean latent period with this method of around 3 minutes.

Neither sperm-egg fusion nor $[Ca^{2+}]_i$ transients occur in Ca^{2+}-free medium

In order to be certain that the sperm taking up the Hoechst 33342 dye were indeed fused, and not merely bound to the surface of the eggs, experiments were carried out on gametes in Ca^{2+}-free media. Fertilization does not occur in media that lack Ca^{2+} and sperm-egg fusion does not take place (Yanagimachi, 1982). When we inseminated eggs in Ca^{2+}-free

Table 2. Incidence of sperm fusion and $[Ca^{2+}]_i$ transient in Ca^{2+}-free medium

	No $[Ca^{2+}]_i$ transient	$[Ca^{2+}]_i$ transient observed
No sperm fusion	100% (6/6)	0% (0/6)
One or more sperm fused	0% (0/6)	0% (0/6)

medium, we observed no uptake of Hoechst dye into the sperm, despite adherence of sperm to the egg surface, nor did we observe any $[Ca^{2+}]_i$ transients (Table 2).

Preventing sperm-egg membrane fusion prevents $[Ca^{2+}]_i$ oscillations

Having established that sperm bound to eggs failed to show transfer of the H33342 dye from the egg, we investigated whether preventing sperm-egg fusion would prevent activation of $[Ca^{2+}]_i$ oscillations. Yanagimachi and others (1980) have shown that sperm-egg fusion is specifically inhibited by low pH medium. Eggs were loaded with H33342 and fura red, as above, and inseminated in low pH medium while monitoring $[Ca^{2+}]_i$. After an hour, eggs were assayed for sperm-egg fusion using the H33342 method, then perfused with normal M2 medium to check for reversibility.

Low pH prevented the onset of $[Ca^{2+}]_i$ oscillations and sperm-egg fusion in 28 out of 30 eggs. Only 2 egg manifested $[Ca^{2+}]_i$ oscillations (Table 3). It was these two eggs that scored positive for fused sperm. The $[Ca^{2+}]_i$ trace from one of the 28 eggs that did not show $[Ca^{2+}]_i$ oscillations is shown in Fig. 5A. On return to normal medium, a further 21 eggs developed $[Ca^{2+}]_i$ oscillations (Fig. 5A) and these 21 also scored positive for fused sperm. All eggs with fused sperm showed calcium oscillations, while no egg was found with one but not the other. This was not a direct affect of pH on the oscillation mechanism, since applying low pH medium to eggs that had already begun $[Ca^{2+}]_i$ oscillations did not alter oscillation frequency (Fig. 5B). These data demonstrate that blocking sperm egg fusion using low pH medium prevents the onset of $[Ca^{2+}]_i$ oscillations.

Although it was reported that low pH medium specifically inhibited sperm egg fusion, it was important to confirm that the treatment was without effect on the sperm acrosome reaction. The effects of incubating capacitated sperm for 1 hour in either normal or low pH M2 medium were compared. Low pH medium did in fact reduce acrosome reaction rates from 26%/hours to 14%/hours. Under the insemination conditions that we used, 10 or more sperm are attached to each egg. The 50% reduction in acrosome reaction rate is not sufficient to account for the substantial inhibition of sperm egg fusion and $[Ca^{2+}]_i$ oscillations that we observed. We also showed that the low pH treatment was not in itself deleteri-

Table 3. Incidence of sperm-egg fusion and $[Ca^{2+}]_i$ release after treatment of eggs with low pH medium

	H33342 transfer observed	$[Ca^{2+}]_i$ transient observed
M2 at pH 6.1	7% (2/30)	7% (2/30)
Return to normal M2	70% (21/30)	70% (21/30)

H33342 fluorescence was examined after the end of treatment (about 1 hour) and at the end of the experiment when normal medium had been perfused onto the eggs.

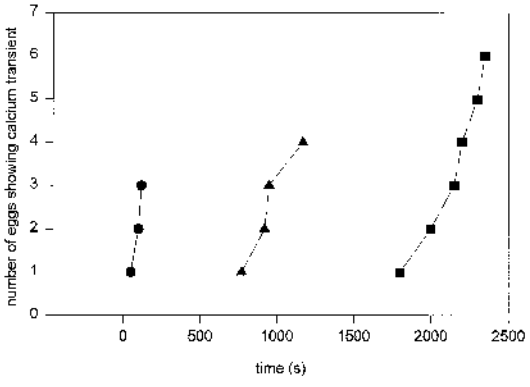


Fig 4. Rate of $[Ca^{2+}]_i$ increase in the egg population in the three experiments in which fertilization rate was constant. The time to onset of the first $[Ca^{2+}]_i$ transient varied markedly and reflects the time it took the sperm to reach the eggs after insemination. Thereafter, the rate of $[Ca^{2+}]_i$ increase, and hence fertilization, was similar.

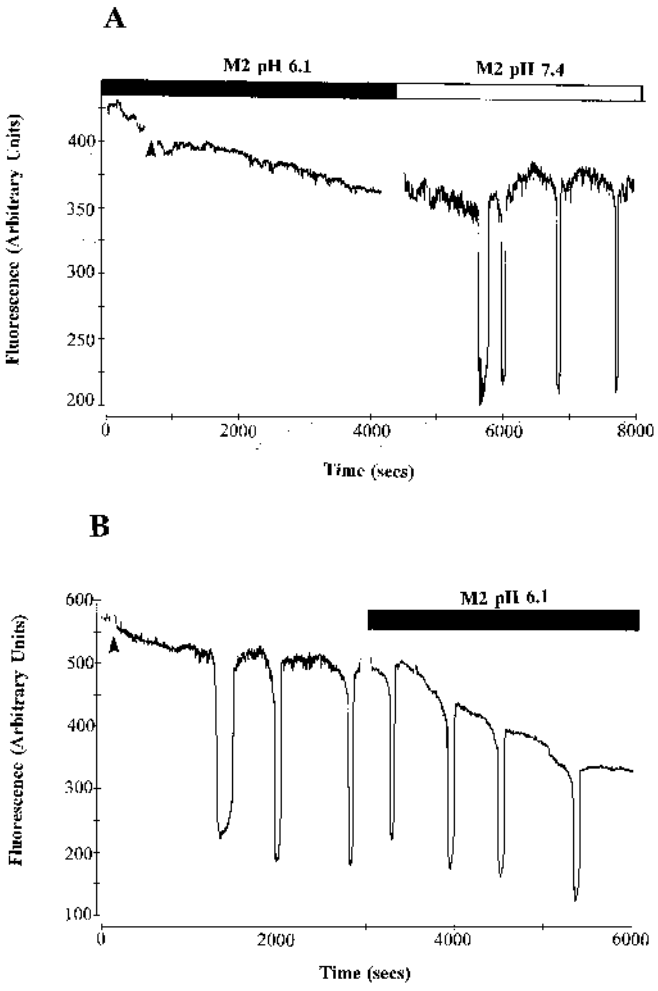


Fig 5. $[Ca^{2+}]_i$ changes in eggs treated with low pH media. $[Ca^{2+}]_i$ was measured with fura red fluorescence as in Fig. 3. In A a recording is shown from an experiment where sperm were added (at the arrowhead) to eggs in medium of pH 6.1 (black bar). After about 1 hour, normal medium (pH 7.4) was perfused into the chamber containing the eggs (white bar). $[Ca^{2+}]_i$ oscillations were seen only after the return to pH 7.4 medium. B shows a typical record from a fertilizing egg that underwent $[Ca^{2+}]_i$ oscillations after addition of sperm (at the arrowhead) to the chamber containing the egg. Subsequent perfusion into pH 6.1 medium was made (black bar) with no effect on the $[Ca^{2+}]_i$ signals. The slow decline in fluorescence in these experiments is due to loss of dye from the egg.

ous to sperm. Fertilization of eggs with sperm pre-treated for an hour at low pH showed that 16/16 eggs displayed $[Ca^{2+}]_i$ oscillations.

It was important to determine whether receptor-mediated signalling was affected in low pH media. The muscarinic antagonist carbachol has previously been shown to induce transient $[Ca^{2+}]_i$ increases in mouse eggs (Swann, 1992). The response of eggs to carbachol in normal and pH 6.1 M2 medium were compared (Fig. 6). They were very similar. In all, 14/14 eggs responded to carbachol in normal medium and 10/10 eggs in low pH medium. This should be compared with the 2/30 eggs that showed $[Ca^{2+}]_i$ oscillations when fertilized in low pH medium.

As a final test that low pH was indeed blocking sperm egg fusion, we compared its effects with those of a well-characterized fusion blocker, Tween 80 (Chernomordik et al., 1993). There are no stereochemically specific blockers of membrane fusion. Agents that have been identified to block fusion are surface-active compounds that intercalate into the lipid bilayer. We chose Tween 80 because it shows the greatest difference between its fusion-blocking concentration and the concentrations at which it manifests its detergent properties. Above its critical micellar concentration (CMC), Tween 80 acts as a detergent membrane disruptor, but below its CMC, it intercalates in the membrane to increase the surface energy associated with intermediate fusion states and blocks fusion (Chernomordik et al., 1993). Tween 80 also reversibly blocked sperm-egg fusion and $[Ca^{2+}]_i$ oscillations (Table 4 and Fig. 7A), though in this case we observed some eggs with fused sperm that did not show $[Ca^{2+}]_i$ oscillations, implying some effect of Tween 80 on the initiation of the $[Ca^{2+}]_i$ oscillation mechanism itself. However, no egg showed $[Ca^{2+}]_i$ oscillations in the absence of a fused sperm. The block was partially reversible: after removal of Tween 80, 14% (8/58) of the eggs underwent sperm-egg fusion and showed $[Ca^{2+}]_i$ oscillations and again, no egg showed any evidence of $[Ca^{2+}]_i$ oscillations in the absence of fusion. Tween 80 did not inhibit $[Ca^{2+}]_i$ oscillations once started (Fig. 7B); if anything, it caused an increase in their frequency. Nor did it abolish receptor-mediated signalling, though it did diminish it: addition of carbachol in the presence of Tween 80 gave $[Ca^{2+}]_i$ oscillations in 8 of 16 eggs (Fig. 7C). This should be compared to the >90% inhibition of sperm egg fusion and $[Ca^{2+}]_i$ oscillations at fertilization. These data closely resemble those we obtained using low pH_i and confirm that sperm-egg fusion is an essential prerequisite of the $[Ca^{2+}]_i$ transients, in that transients were never observed in the absence of sperm-egg fusion.

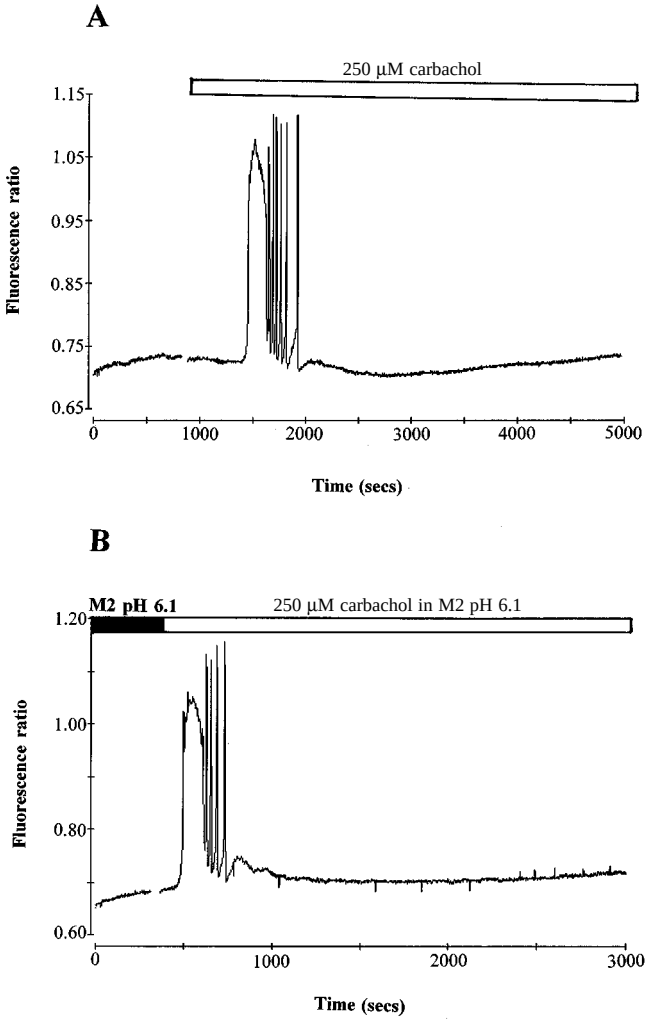


Fig 6. $[Ca^{2+}]_i$ changes in eggs in response to the muscarinic agonist carbachol. $[Ca^{2+}]_i$ was monitored by fura red fluorescence which is shown as a ratio of the 440:490 nm excitation intensities. (A) The egg in normal M2 medium was stimulated with a final concentration of 250 μM carbachol. (B) The egg was kept continuously in pH 6.1 medium and stimulated by carbachol addition (the white bar).

Table 4. Incidence of sperm-egg fusion and $[Ca^{2+}]_i$ release after treatment of eggs with Tween 80 medium

	H33342 transfer observed	$[Ca^{2+}]_i$ transient observed
0.25% Tween 80	12% (7/58)	2% (1/58)
Return to normal M2	26% (15/58)	14% (8/58)

H33342 fluorescence was examined after the end of treatment (about 1 hour) and at the end of the experiment when normal medium had been perfused onto the eggs.

DISCUSSION

Using two independent approaches, we have shown that sperm-egg fusion precedes the onset of $[Ca^{2+}]_i$ oscillations in mouse eggs and that fusion is independent of increases in $[Ca^{2+}]_i$. We have also shown that treatments that block sperm-egg fusion prevent $[Ca^{2+}]_i$ oscillations developing.

Dye transfer and $[Ca^{2+}]_i$ increases

It is clearly possible to demonstrate the transfer of fluorescent dyes from the mouse egg into the sperm at fertilization. Both a dextran coupled $[Ca^{2+}]_i$ sensitive dye and the DNA-staining Hoechst dye can diffuse from the egg into the sperm before the start of the first $[Ca^{2+}]_i$ increase during fertilization. The precise time difference between the transfer of dye and the onset of the initial $[Ca^{2+}]_i$ transient could not be defined using confocal microcopy of Ca^{2+}-green dextran-injected eggs because the time of detection of dye transfer to sperm repre-

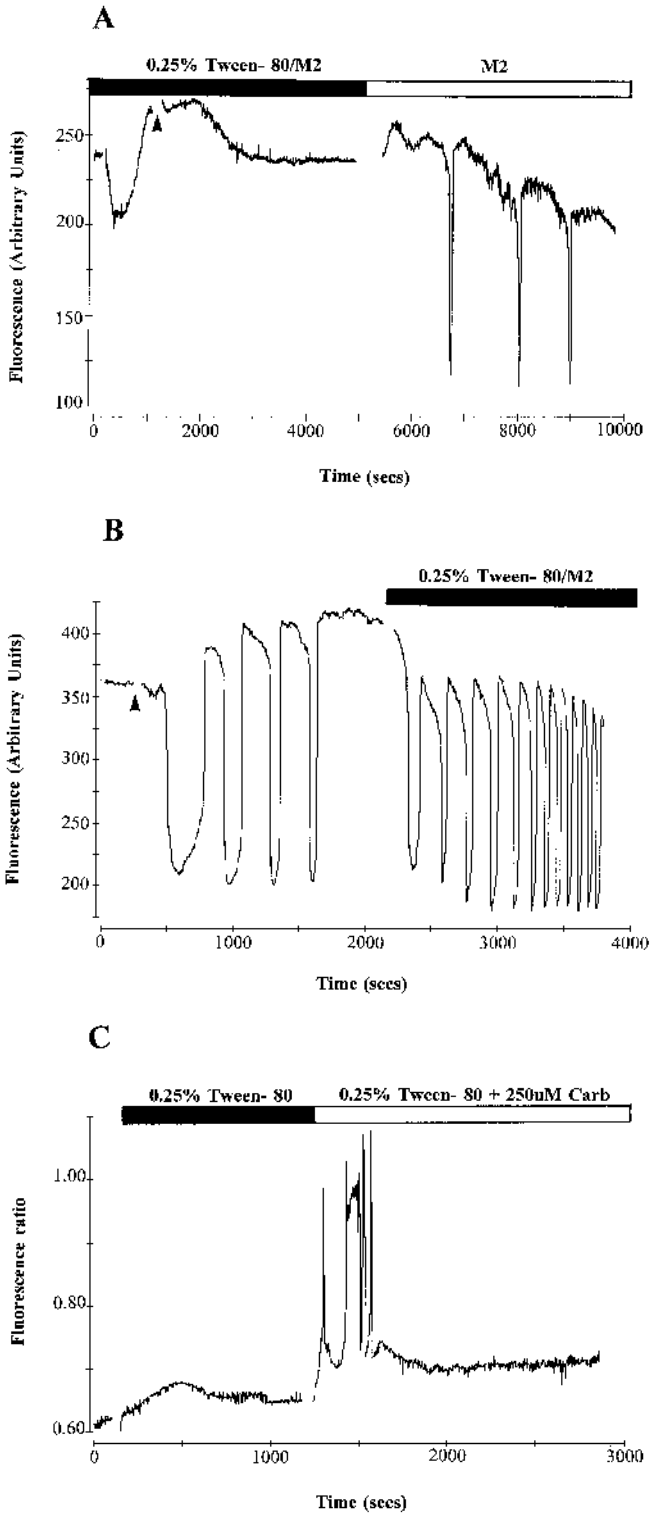


Fig. 7. $[Ca^{2+}]_i$ transients recorded in eggs treated with Tween 80 medium. In A a recording is shown from an egg in M2 medium plus 0.25% Tween 80. A slight increase in resting $[Ca^{2+}]_i$ occurred due to the Tween 80 medium itself, but no $[Ca^{2+}]_i$ oscillations where seen after addition of sperm (at the arrowhead). However, when normal medium was perfused onto the egg (white bar) the egg underwent $[Ca^{2+}]_i$ oscillations. In B is shown a typical recording from an egg undergoing $[Ca^{2+}]_i$ oscillations after addition of sperm (at the arrowhead). The egg was then perfused with M2 medium containing 0.25% Tween 80 (black bar). In C the egg was bathed continuously in M2 medium plus 0.25% Tween 80 and then stimulated with 250 μM carbachol (the white bar).

sents in part the variable time after fusion that it takes for the experimenter to identify a fluorescent sperm by scanning up and down through the egg. However it is clear that both the transfer of dextran-coupled dyes and H33342 can occur several minutes before and independent of the first $[Ca^{2+}]_i$ increase. The experiments that measured fusion with H33342 and $[Ca^{2+}]_i$ with fura red also showed that a $[Ca^{2+}]_i$ increase is always associated with gamete fusion, which suggests that fusion always occurs before the first $[Ca^{2+}]_i$ increase.

We also demonstrate that gamete fusion does not require a $[Ca^{2+}]_i$ increase, using BAPTA buffers. In fact the number of sperm that fused to BAPTA-injected eggs (3.6) is high considering the sperm densities used. This may indicate a permissive role for $[Ca^{2+}]_i$ in blocks to polyspermy since in independent experiments using BAPTA-AM, loaded eggs have indicated an increase in entry of sperm over controls (Lawrence and Swann, unpubished observations). In eggs that were not loaded with BAPTA, we were able to make a crude estimate of 1-3 minutes for the delay between dye transfer and the onset $[Ca^{2+}]_i$ increase by using H33342 and fura red. Any slight differences in the times we measured for the delay between the Ca^{2+}-green dextran and H33342/fura red experiments could be methodological since we have detected slight differences in the pattern of $[Ca^{2+}]_i$ oscillations at fertilization in mouse eggs depending upon the dye used, ie dextran coupled fura 2 versus fura 2-AM (Lawrence and Swann, unpublished data). Regardless, our current observations clearly demonstrate that the $[Ca^{2+}]_i$ oscillations that lead to the activation of mouse eggs at fertilization are generated after the sperm and egg membranes have fused. They also suggest that sperm-egg membrane fusion is a prerequisite for the initiation of $[Ca^{2+}]_i$ oscillations at fertilization.

In sea urchin eggs it has been shown that the time between sperm-egg continuity and the $[Ca^{2+}]_i$ wave, which is equivalent to the latent period, is around 10-15 seconds (McCulloh and Chambers, 1992; Whitaker and Swann, 1993). Our data suggest that a somewhat longer latent period, in the order of minutes, exists during fertilization in mouse. This is consistent with previous observations on the timing of sperm-egg interaction and indications of the first $[Ca^{2+}]_i$ increase in mouse and other mammals. A clear indication that a sperm is going to fertilize an egg is the cessation of tail motility of an attached sperm (Miyazaki and Igusa, 1981; Igusa et al., 1983; Yanagimachi, 1994). The reaction may be caused by gamete fusion, although this remains to be shown. Nevertheless, the observations on sperm motility cessation are consistent with our data. The average time between sperm tail motility cessation and small membrane potential hyperpolarizations, which probably reflect $[Ca^{2+}]_i$ increases, has been reported to be 7 minutes in the mouse, with membrane potential changes occurring as early as 2 minutes after the sperm tail stops beating (Jaffe et al., 1983). Our findings are also consistent with a delay of about 5-10 minutes between sperm addition to zona free mouse eggs and the start of exocytosis or $[Ca^{2+}]_i$ oscillations (Lee et al., 1988; Kline and Kline, 1992; Swann and Ozil, 1994). Some

differences in temperature may account for potential variability in mouse eggs since the latent period appears to be rather temperature dependent (Allen and Griffen, 1958). However, the relatively long latent period in mouse is not a feature of all mammals since the time between sperm tail motility cessation and the first Ca^{2+} increase is likely to be less than 10 seconds in hamster fertilization (Miyazaki and Igusa, 1981; Miyazaki et al., 1993). The differences within mammals may be due to the sperm, since the time that elapses from when the sperm tail stops beating until the initial $[Ca^{2+}]_i$ release is longer when mouse sperm fuse with hamster eggs than when hamster sperm fuse with hamster eggs (Igusa et al., 1983).

These dye transfer experiments demonstrate that molecules can move rapidly between sperm and egg immediately after membrane fusion and before $[Ca]_i$ oscillations begin. This is a necessary condition for the hypothesis that an activating molecule passes from sperm to egg (Swann, 1990).

Preventing sperm-egg fusion

Previous experiments in hamsters have shown that low pH media inhibit sperm-egg fusion (Yanagimachi et al., 1980). We have demonstrated that low pH inhibits sperm-egg fusion in the mouse and is tightly correlated with the inhibition of the onset of $[Ca^{2+}]_i$ oscillations. The inhibition of fusion and $[Ca^{2+}]_i$ signals was reversible. Furthermore, low pH did not inhibit $[Ca^{2+}]_i$ oscillations once they had begun in eggs. This suggests that it is only the initiation of $[Ca^{2+}]_i$ oscillations that is affected by the inhibition of gamete fusion. It should be borne in mind that inhibitors of gamete fusion may also be affecting a receptor linked mechanism that generates both membrane fusion and $[Ca^{2+}]_i$ signals. This is difficult to test specifically as yet, since no sperm bound agonists that trigger $[Ca^{2+}]_i$ changes in mammalian eggs have been indentified. One way of stimulating $[Ca^{2+}]_i$ changes via a receptor is to add carbachol to eggs (Swann, 1992). Since this pathway was not affected by low pH, the effect of low pH on fertilization cannot readily be explained by a general disruption of receptors.

We have demonstrated that low pH is indeed blocking fusion, not the sperm acrosome reaction. We have also compared the effects of low pH with those of a well-characterized blocker of membrane fusion, Tween 80, the best available for blocking fusion between external lipid leaflets in living cells (Chernomordik et al., 1993) The data with the two blockers are comparable, though Tween 80 had a tendency to supress the initiation of $[Ca^{2+}]_i$ signals. These observations confirm that low pH is blocking at the sperm-egg fusion step.

Mechanism of egg activation

The fusion between eggs and sperm in mammals appears to be mediated via a specific sperm protein, fertilin, and integrin receptors on the egg membrane surface (Almeida et al., 1995). The question is whether the integrin receptor independently induces both sperm-egg fusion and the postfertilization $[Ca^{2+}]_i$ oscillations or whether fusion itself generates the $[Ca^{2+}]_i$ signals. It is known that sperm contain a protein, oscillin, that is capable of generating $[Ca^{2+}]_i$ oscillations identical in their proprties to those recorded after fertilization (Parrington et al., 1996).

We show here that sperm-egg fusion is independent of changes in $[Ca^{2+}]_i$, thus eliminating the possibility that it is a consequence of receptor-induced $[Ca^{2+}]_i$ oscillations. We find that sperm-egg fusion occurs before the $[Ca^{2+}]_i$ transient is initiated and that $[Ca^{2+}]_i$ transients are never observed, under any conditions, in the absence of sperm-egg fusion. Consequently, the simplest explanation of these data that we can offer is that the initiation of $[Ca^{2+}]_i$ oscillations at fertilization is due to sperm-egg membrane fusion.

We cannot formally exclude the possibility that the agents that we have used to inhibit sperm egg fusion may affect the fertilin/integrin receptor mechanism, rather than fusion itself. Other integrin receptors generate $[Ca^{2+}]_i$ transients in response to RGD peptide (Evans et al., 1995). The mouse egg integrin receptor does not respond to the same concentrations of this peptide (J. Carroll, personal communication), though the peptide blocks sperm egg binding at these concentrations (Evans et al., 1995). Nor does the fertilin/integrin receptor couple appear to initiate any $[Ca^{2+}]_i$ signals (Schultz and Kopf, 1995). This is perhaps an argument against a direct role for the mouse egg integrin receptor in generating $[Ca^{2+}]_i$ transients, but, in any event, it prevents us from testing the effects of the fusion inhibitors on the putative signalling function of the integrin receptor directly. Nonetheless, we have shown that the acetylcholine receptor signal transduction mechanism is not affected by conditions that inhibit fusion. We have also demonstrated that $[Ca^{2+}]_i$ transients do not occur in the absence of one or more fused sperm. This apparently indissoluble link between fusion and the generation of $[Ca^{2+}]_i$ transients indicates that there is a strong mechanistic link between fusion and egg activation, irrespective of specific hypotheses of signal transduction. It can be interpreted as a fail-safe mechanism that ensures the delivery of the paternal genome along with the activation signal. Whether this link represents the diffusion of oscillin from sperm to egg remains to be seen, though our observations point in that direction.

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