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Function and characteristics of repetitive calcium waves associated with meiosis

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Function and characteristics of repetitive calcium waves associated with meiosis

[Research Paper]

Alex McDougall, Christian Sardet

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Abstract

Background

Internal calcium waves and oscillations are now recognized as universal features of cellular activation, but their exact role remains uncertain. In mammalian and ascidian eggs, a large triggered calcium activation wave crosses the egg at fertilization, followed by a series of pe increases in intracellular calcium concentration ($[Ca^{2+}]_i$). We have previously shown that, i the ascidian *Phallusia mammillata*, these periodic, post-activation $[Ca^{2+}]_i$ increases are in t of waves, the origin of which relocalizes to a pacemaker region, and that they stop seconds the completion of meiosis.

Disculte

RESULTS

We show here that the origin of the first one to four post-activation calcium waves in *P. ma* eggs transfers progressively from the site of sperm entry, usually in the animal hemisphere towards an endoplasmic reticulum (ER)-rich contraction pole in the vegetal hemisphere, a process that takes about five minutes. Once the origin of these repetitive post-activation calcium waves reached the contraction pole, all subsequent calcium waves originate from the domain of ER concentrated there, which acts as a pacemaker. The first few post-activation calcium waves faster than the activation wave and, like the activation wave, they propagate homogeneous throughout the cytoplasm. Approximately five to ten minutes after fertilization, the post-activation calcium waves begin to propagate preferentially in the egg cortex. By manipulating intracellular calcium levels with caged inositol 1, 4, 5 trisphosphate (InsP₃) and a competitive inhibitor of calcium-induced calcium release, we show that the activation wave induced by the sperm is sufficient to induce extrusion of the first polar body, but that additional [Ca²⁺]_i increases are necessary for completion of the second meiotic division. However, periodic calcium waves *per se* do not seem to be strictly necessary for the completion of meiosis, as a persistent and homogeneous increase in calcium, induced by the calcium ionophore ionomycin, is sufficient to cause second polar body formation and allow completion of meiosis on time.

Conclusion

These results clearly show that, in the ascidian egg, post-activation calcium waves are required to complete meiosis. They also show that following a period of progressive relocalization of the origin, which lasts approximately five minutes, an ER-rich domain at the contraction pole first becomes a pacemaker from which the calcium waves originate. Once their origin becomes spatially localized, the calcium waves begin to propagate preferentially around the cortex of the egg rather than throughout the egg cytoplasm.

Background

Calcium waves are a central feature of fertilization in many deuterostome eggs [1] and, in other cells, such waves and oscillations are at the heart of several signal transduction processes [4]. Reinitiation of the cell cycle in the eggs of most deuterostomes (sea urchins, ascidians, amphibians, fish and mammals, for example) is accomplished at fertilization by a sperm-triggered increase in the level of free internal calcium ([Ca²⁺]_i) [1] [5] [6] [7] [8] [9]. Although the increase of many deuterostome eggs stimulates just a single calcium wave [8] [9] [10], in mammalian ascidian eggs, fertilization has been shown to be followed by a series of repetitive [Ca²⁺]_i increases in addition to the activation wave [5] [11] [12] [13].

In contrast to mammalian eggs, where most periodic [Ca²⁺]_i increases appear synchronous throughout the egg cytoplasm [13], the periodic [Ca²⁺]_i increases in eggs of ascidians (such as *Phallusia mammillata*) occur in the form of waves [14]. The first, and activating, calcium wave is triggered preferentially in the animal hemisphere by the sperm that enters the egg there. Following this activating wave, a series of 12–25 smaller and faster calcium waves cross the egg [15]. These waves originate predominately in the opposite, vegetal hemisphere [15]. The activating calcium wave presumably induces the contraction of a cortical actomyosin basket (open arc in the animal pole region), thereby stimulating the first phase of ooplasmic segregation [6] [17] [18]. Besides concentrating a mitochondria-rich domain (the myoplasm) in the vegetal hemisphere [19], this contraction also induces an endoplasmic reticulum (ER)-rich domain to form in the animal hemisphere at a 'contraction pole' [20] [21]. Although, in these experiments, the lateral reorganization obtained by photon imaging of aequorin luminescence was limited (to around 10–20 μm²), the observed stratification of mitochondria away from the contraction pole strongly suggested that post-activation calcium waves probably originate from this domain of ER [16].

Instead of using aequorin, we have used a confocal microscope to image the light signal emitted from two fluorescent dyes in 1–2 μm-thick optical sections, and thus improved the spatial resolution. As cytoplasmic domains composed of distinct organelles form in the ascidian egg minutes after fertilization [18] [21], the use of just one calcium dye could be misleading, as the cytoplasmic space varies in the different domains. To overcome this dye-distribution problem, we have simultaneously injected two 10 000 D molecular weight, dextran-coupled fluorescent calcium indicators, calcium green-1 (CG) and Texas Red (TR) — into the egg. The much larger dextran ensures cytoplasmic distribution of both dyes. Also, as TR is calcium-insensitive and CG is calcium-sensitive, we determine the ratios of simultaneously acquired confocal images in real time. This approach removes potential artifacts, including dye internalization and dye-buffering of internal calcium, by using relatively low intracellular concentrations of CG [22]. By using confocal microscopy, we have been able to improve our spatial resolution to around 2–5 μm in the x–y plane and 1–2 μm in the z plane, allowing precise characterization of the spatial aspects of these periodic calcium waves.

It is not clear what role the post-fertilization calcium signals have on meiosis, but it has been

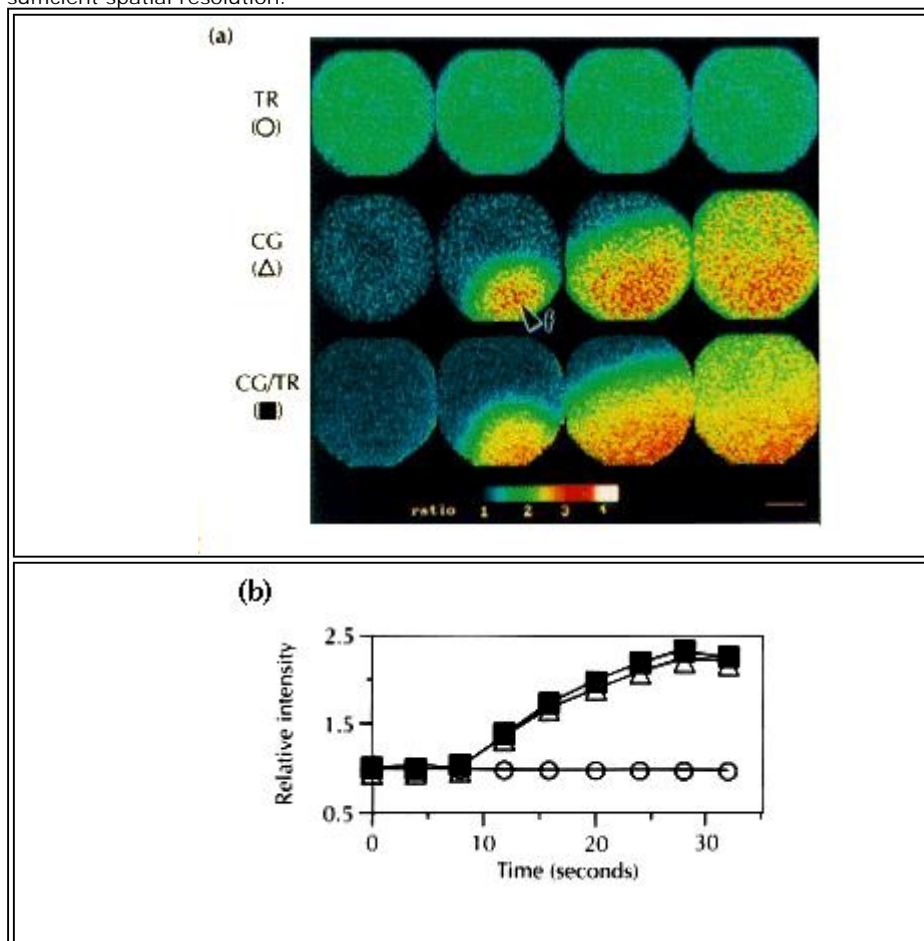
It is not clear what role the post-fertilization calcium signals have on meiosis, but it has been recently that an increase in $[Ca^{2+}]_i$ has to occur simultaneously with the appearance of an spindle for eggs to exit M-phase [23]. In mouse eggs, exit from the meiotic metaphase II is brought about by a drop in the level of cyclin B, and consequent inactivation of M-phase promoting factor (MPF) [23] [24]. In *Xenopus* eggs, the calcium-induced activation of calmodulin kinase (CaMKII) induces the requisite cyclin destruction [25]. Indeed, if constitutively active CaMK overexpressed in the yeast *Schizosaccharomyces pombe*, the p34^{cdc2} kinase that is the act component of MPF is not activated, and the cells arrest in G2 [26]. Internal calcium signals occur before nuclear envelope breakdown and before the onset of anaphase in mitosis [27] [29] [30] [31], which may be triggered by protein-synthesis-independent, but cell-cycle-dependent oscillations in the $InsP_3$ level [32]. These data suggest that the cyclin destruction machinery activated by the calcium-induced activation of CaMKII. The post-activation $[Ca^{2+}]_i$ increase occur at fertilization may even influence later developmental events as, in mouse eggs, artificially induced periodic $[Ca^{2+}]_i$ increases apparently affect developmental potential after the third [33].

Ascidian eggs are arrested at the first meiotic metaphase. It takes about 25 minutes to complete meiosis after fertilization; during this time, a series of calcium waves crosses the egg, and a second polar body is immediately extruded when the calcium waves stop [5] [14]. This correlation has led us to suggest that these periodic calcium waves play a role in completing meiosis II [5] [14]. We have now examined this possibility by inhibiting these calcium waves with the known $InsP_3$ receptor channel-blocker [34], and inducing meiosis by artificially raising the $[Ca^{2+}]_i$ of the ascidian *P. mammillata*.

Results

Imaging free intracellular calcium in ascidian eggs

We imaged calcium in ascidian eggs using the fluorescent calcium dye CG coupled to dextran. We used low-light-level video imaging or confocal microscopy. Fig. 1a shows confocal images of the activating wave in fertilizing *P. mammillata* eggs. The CG, TR and ratio (CG/TR) images are shown. The activating calcium wave passed through the centre of the egg and propagated essentially at a constant speed throughout the cytoplasm. Fig. 1b shows the pixel intensity of the experiment displayed in Fig. 1a. This indicates that, under our experimental conditions, the calcium-sensitive CG signal can be isolated from the calcium-insensitive TR signal. We can therefore determine the CG/TR ratio, which gives us a good quantitative estimate of the internal calcium concentration with sufficient spatial resolution.



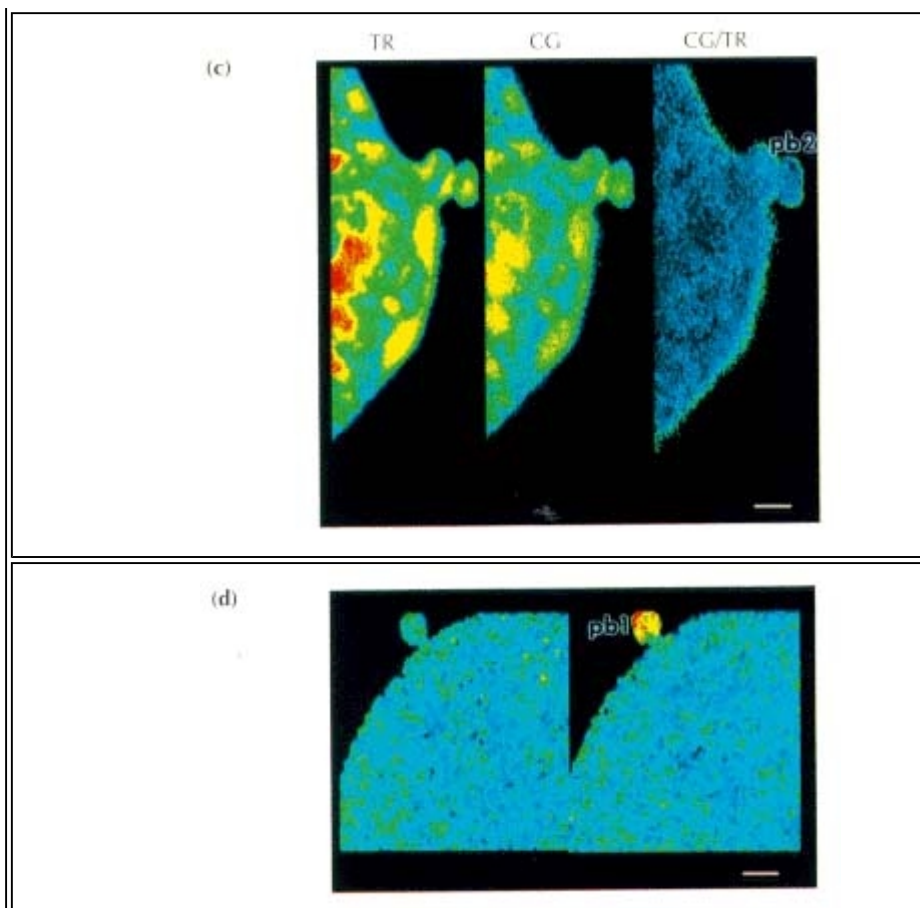


Fig. 1. Confocal ratio imaging of calcium with dyes CG and TR. These confocal optical sections were all $\sim 1\text{--}2\ \mu\text{m}$ thick. The data were collected at $18\text{--}21\ ^\circ\text{C}$. Dechorionated oocytes of the ascidian *P. mammillata* were co-injected with 10 000 D dextran-coupled TR and CG (10–2 and fertilized 30 min later. Images were collected every 2 sec by confocal laser scanning microscopy (CLSM). **(a)** The activating wave (arrowhead *f*) in a *P. mammillata* egg that has been fertilized. TR, CG and ratio (CG/TR) images are shown. The activation wave at fertilization propagates through the egg cytoplasm. The ratios, rather than the calibrated calcium data shown, as CG is not well suited to report the very high ($7\ \mu\text{M}$) calcium levels known (from aequorin data [5]) to accompany the activation wave. Scale bar = $40\ \mu\text{m}$. **(b)** The relative intensities of the CG and TR fluorescent emissions from (a) against time. Open triangles represent CG fluorescence; open circles TR fluorescence; and filled squares the ratio data (CG/TR). This included simply to demonstrate that there is no spillover between the recording channels. **(c)** An egg extruding the second polar body (pb2) about 20 min after fertilization, when the repetitive calcium waves had just ceased. The TR, CG and ratio (CG/TR) data are displayed. This image clearly shows distinct cytoplasmic domains that contain various amounts of the dextran-coupled dyes. The ratio image (CG/TR) shows that the domains disappear, as one would expect if the TR are present at the same concentration in the cytoplasmic space of each domain (scale bar $1\ \mu\text{m}$). **(d)** To obtain an estimate of the spatial resolution ($x\text{--}y$ plane), we imaged the first polar body. This egg was co-injected with CG and TR before extrusion of the first polar body. As we detect changes in the internal calcium level in the polar body, which measures $4\ \mu\text{m}$ in diameter, we know we can resolve $[\text{Ca}^{2+}]_i$ increases within an area this small (scale bar = $5\ \mu\text{m}$).

Return to text reference [1] [2] [3] [4] [5] [6] [7] [8]

One important reason to perform the CG/TR ratio is illustrated by Fig. 1c, in which the egg is completing meiosis and extruding the second polar body. In the fertilized ascidian egg, there are distinct domains of cytoplasm in which the composition of organelles varies; these domains contain varying amounts of the dextran-coupled dyes. If we used only CG, the bright areas could be misinterpreted as regions where the resting internal calcium concentration is higher. As the ratio image ensures an equal distribution of both dyes in the cytoplasmic space, one would expect these hotspots to disappear in the ratio images if they simply reflect an increased amount of dye volume resulting from the larger cytoplasmic space available to the dye in some domains. The ratio image in Fig. 1c shows that this is the case. This not only confirms that the dyes are concentrated in certain cytoplasmic domains, but it is also good indirect evidence that, after microinjection, there is an equal distribution of both of the dextran-coupled CG and TR dyes in the cytoplasm.

We obtained similar results with a dextran (70 000 D, courtesy of Molecular Probes) that has both the CG and TR dyes coupled to it (with a 1:1 molar ratio of both dyes per dextran). To estimate the resolution in the $x\text{--}y$ plane, we injected eggs before fertilization and imaged the resting internal calcium level in the polar body (which is $\sim 4\ \mu\text{m}$ in diameter). In some cases, the polar body internal calcium level oscillated out of phase with the egg. This allowed us to determine if we could resolve $[\text{Ca}^{2+}]_i$ increases in small cytoplasmic areas using our ratiometric methodology. As

observe $[Ca^{2+}]_i$ increases in small cytoplasmic areas using our ratiometric methodology. We are able to detect $[Ca^{2+}]_i$ increases within the polar body which persisted in the ratiometric images (Fig. 1d), we infer that our horizontal resolution is somewhere in the range of 2–4 μm . This resolution is required, as the domain of ER at the contraction pole, suspected to be the origin of calcium waves [16], has been measured to be about 2–6 μm thick [21].

A similar fraction of the injected and uninjected oocytes developed normally to tadpoles over our experimental conditions. We could detect $[Ca^{2+}]_i$ increases as the tadpole beat ($n = 7$), indicating that the injected dextran-coupled CG remained cytoplasmic and functional (blastomeres divided and the embryo differentiated (data not shown)).

The periodic post-activation calcium waves become progressively localized to a pacemaker region

Fertilization of oocytes with pre-activated sperm results in a large $[Ca^{2+}]_i$ increase that crosses the egg as a wave from the site of sperm–egg fusion ([14] [35] and Fig. 1a). Using aequorin, it has been shown that the peak $[Ca^{2+}]_i$ increase at activation is $\sim 7 \mu\text{M}$, whereas the later post-activation calcium waves are 1–4 μM [5]. We have confirmed that all the post-activation $[Ca^{2+}]_i$ increases travel as waves and that their peak is around 2 μM . We had previously noted that the first periodic calcium waves originated in the animal hemisphere and the later waves principally originated from the vegetal pole region [14]. The improved spatial resolution attainable with fluorescent dyes has allowed us to show that the origin of the post-activation calcium wave indeed moves to the vegetal hemisphere, but it does so slowly and progressively around the cortex (Fig. 2). There are normally between one and four ($n = 9$) of these progressively localized calcium waves, depending on where the sperm enters the egg (see Fig. 2 and Fig. 4a) and where the activation wave originates. In the case of the egg shown in Fig. 2, the activating wave (the row of images marked 'act') was followed by a series of three progressively localized waves (see waves marked 1–3).

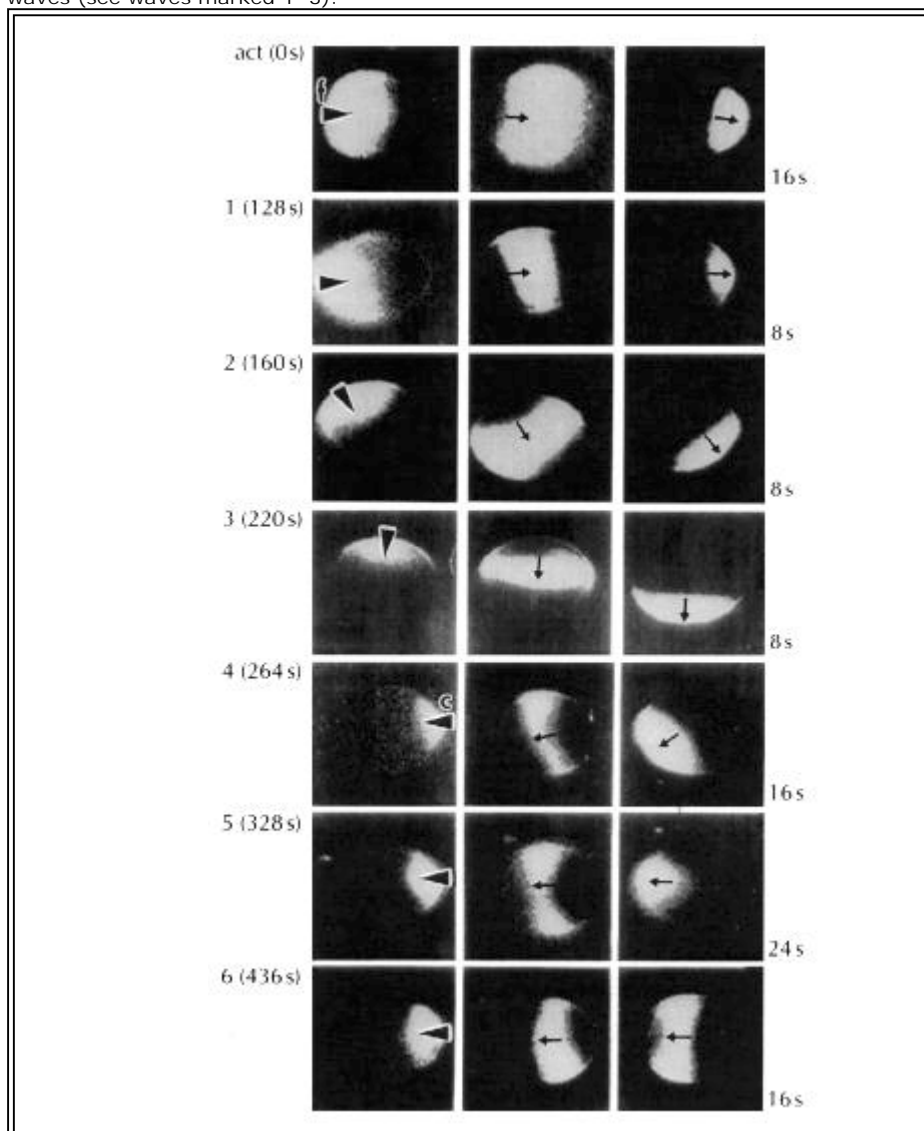


Fig. 2. Progressive localization of post-activation calcium waves. Dechorionated oocytes w

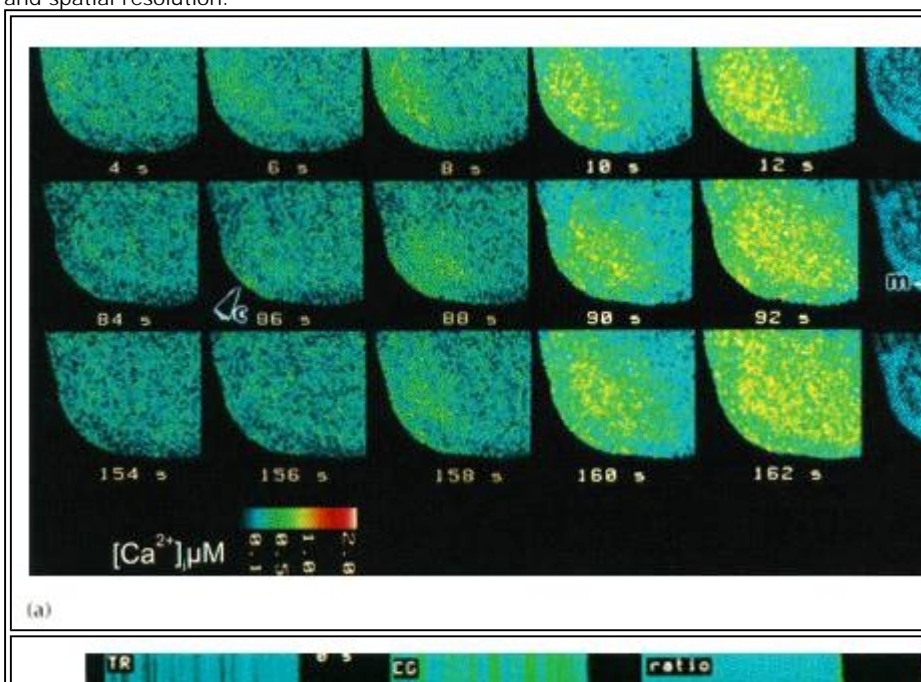
injected with 10 000 D-dextran-coupled CG (10–20 μM) and fertilized 30 min later. Image collected at 4 sec intervals using a SIT camera, the Image 1 software and an OMDR to store images. The images are displayed to indicate $[\text{Ca}^{2+}]_i$ increases as clearly as possible — by subtracting previous images ($n - (n-1)$), the sites of initiation (arrowheads) of the waves and direction of propagation (arrows) are visualized most clearly. The first seven calcium waves from the same egg are shown, beginning with the first, activating wave (the row of images marked 'act') that originates from the site of fertilization (arrowhead f). The periodic calcium waves that follow are numbered 1–6, and their sites of origin are indicated (arrowheads), as are the times at which the waves begin. The site of origin becomes progressively localized following fertilization (compare waves 1–3 to waves 4–6; there are, on average, 1–4 post-activation waves before localization occurs) to the contraction pole (labelled c) and the direction of propagation (arrows) reflects this progressive localization. The time to cross the egg is shown on the right: note this initially decreases (waves 1–3) then increases (waves 4–6). This later increase may reflect preferentially cortical propagation (see text and Fig 4). All subsequent calcium waves come from this contraction pole ($n = 9, 18-21$ °C).

Return to text reference [1] [2] [3] [4] [5] [6] [7] [8] [9] [10]

The calcium waves became stably localized 4–5 minutes after fertilization (waves 4–6 in Fig. 2). The movement of this early pacemaker was slower, taking about 5 minutes, than the reported movement of the cortical contraction (measured by the movement of attached surface particle which took about 3 minutes [18]). Indeed, in one experiment where we had surface particle attached to the egg, we were fortunate enough to observe fertilization and the onset of a calcium wave right under a particle (near, but not exactly on, the animal pole). In this case we clearly saw that the surface particle was swept towards the vegetal hemisphere and the contraction pole before the calcium waves localized to the same region (data not shown). This demonstrates that the early pacemaker probably does not rely upon a factor localized at the level of the plasma membrane. Once stably localized, the calcium waves clearly originate from a specialized region, the contraction pole (marked c in Fig. 2, wave 4). The contraction pole is a cell-surface protrusion situated within 50 ° of the vegetal pole, and is the result of a cortical contraction triggered by a calcium activation wave [6]. We previously suggested that the late, stable pacemaker consists of cytoplasmic domains situated in the contraction pole [14] [20] [21], and experiments based on the relocation of cytoplasmic domains make this a very likely possibility [16].

The ER-rich domain in the contraction pole becomes the calcium waves' pacemaker

To determine whether the later, post-activation calcium waves originate precisely from the thick domain, consisting almost entirely of densely stacked ER tubes and sheets, underlying plasma membrane in the contraction pole [21], we co-injected CG- and TR-coupled dextran into the egg and subsequently imaged ~ 1 μm -thick, equatorial confocal sections in the contraction pole while repetitive calcium waves originated from this region. Fig. 3a shows the onset of three successive post-activation calcium waves that originated from the contraction pole (marked in the second row of images in Fig. 3a) just as the calcium waves first became stably localized (~ 5 minutes after fertilization). In this figure, ratio images (CG/TR) are shown together with TR images to identify the presence of distinct cytoplasmic domains in the confocal sections. In order to identify the onset of the periodic post-activation calcium waves, we recorded several successive waves at high magnification, while precisely adjusting the level of the section to localize the pacemaker. We scanned every 2 seconds, which gave a good compromise between time and spatial resolution.



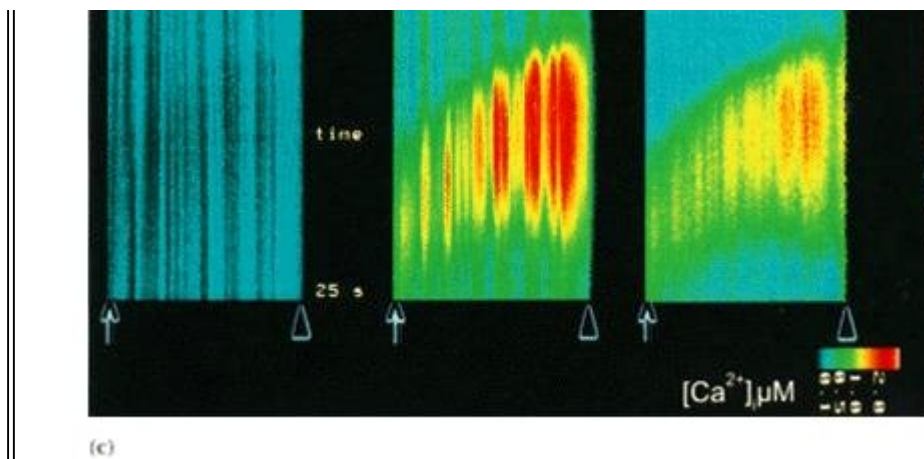


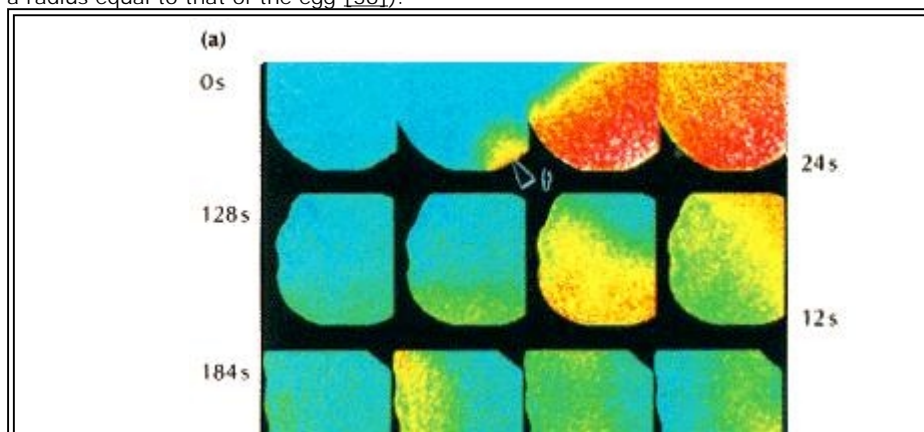
Fig. 3. An ER-rich domain is the calcium-wave pacemaker. Dechorionated oocytes were co-coupled TR and CG (10–20 μM) and later fertilized. Images were collected every 2 sec using confocal images of three successive calcium waves that come from the same area in the cell (18–21 $^{\circ}\text{C}$). The five ratio images in each scene are shown, together with a TR image (panel showing the cytoplasmic domains in that region (m, myoplasm or mitochondria-rich domain) using a zoom function, the area from which the periodic waves come has been expanded (analysis indicates that the $[\text{Ca}^{2+}]_i$ increase first occurs (arrowhead) in the contraction pole plasma membrane (arrowhead). The $[\text{Ca}^{2+}]_i$ increase begins at the level of the plasma membrane domain of concentrated ER resides ($n = 6$, 21 $^{\circ}\text{C}$). Note also the overall lower $[\text{Ca}^{2+}]_i$ increase domain (marked m) as the calcium wave is triggered close to the plasma membrane. (c) increase in the contraction pole area further, we used the line-scanning mode of the confocal through the contraction pole (marked c), as illustrated in the TR image on the extreme right ($\times 256$ format) shown on the left took 26 sec to accumulate. The scan-line passed through average of 32 scans). Each 100 msec, the scanned line was stacked vertically in descending vertical axis in this panel. This further improves both the lateral resolution and the temporal origin of the calcium wave is immediately beneath the plasma membrane (large arrowhead

Return to text reference [1] [2] [3] [4] [5] [6] [7]

Careful quantitative analysis of the second sequence in Fig. 3a (the rectangular boxes in Fig. 3a display the pixel intensity in that area) showed that the $[\text{Ca}^{2+}]_i$ increases first in the contraction pole at a discrete site just beneath the plasma membrane, about 2 seconds before it is seen in neighbouring regions (Fig. 3b). We could improve the temporal resolution of these measurements further by scanning a single line, passing through the site of origin of the later post-activation calcium waves, that began in the contraction pole. The TR, CG and ratio (CG/TR) images are in Fig. 3c, together with a TR image that shows where the scanline passed through the egg ratio data confirm that the post-activation waves start near the plasma membrane, where the ER domain is located. These experiments show that the cortical ER domain at the contraction pole constitutes the late and stably localized pacemaker of the post-activation calcium waves.

Late periodic post-activation calcium waves propagate preferentially in the egg cortex

The activating calcium wave clearly propagated evenly, with a uniform velocity, throughout cytoplasm (see Fig. 1a and Fig. 4a). The wavefront reached the opposite side of the egg essentially a flat wavefront, which is exactly as predicted if the wave propagates at constant velocity throughout the cytoplasm, creating a wave consisting of two spherical caps (the leading spherical cap having an increasing radius equal to the distance travelled into the egg, the trailing one a radius equal to that of the egg [36]).



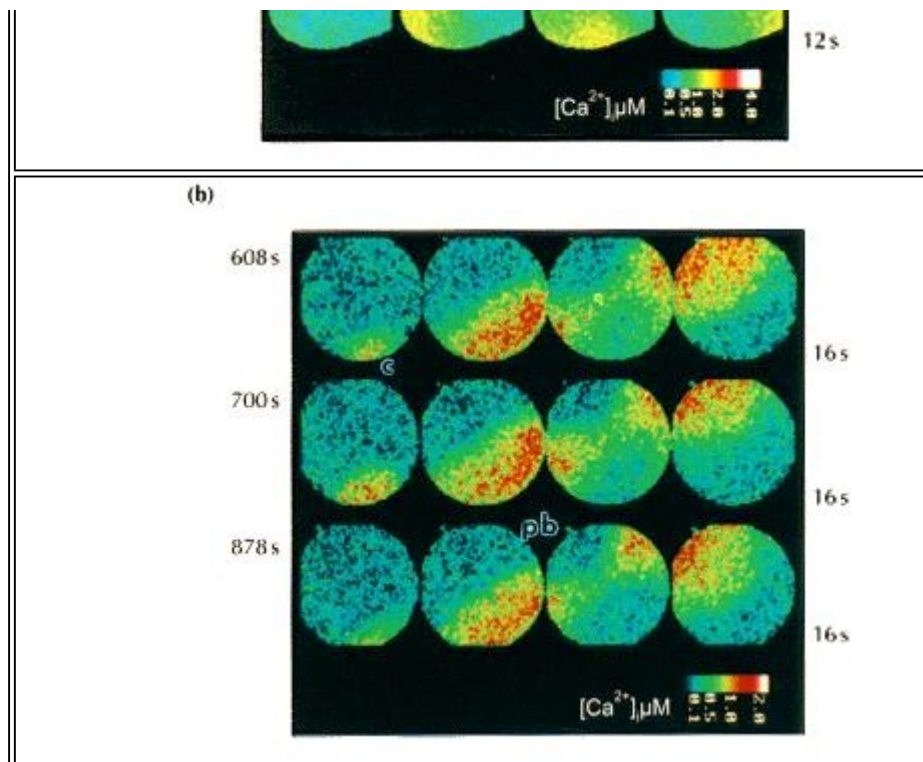


Fig. 4. Cytoplasmic and cortical calcium waves. Dechorionated oocytes were co-injected with 1000 D-dextran-coupled CG and TR (10–20 μM) and fertilized 30 min later. Ratio images ($[\text{Ca}^{2+}]$) were collected every 2 sec using CLSM. **(a)** The activation calcium wave (act) travels through the centre of the egg from the site of sperm–egg fusion (arrowhead), crossing it in 24 sec. The next row of images shows the next wave in this egg, which is a progressively localizing one; this also travels through the centre of the egg, but is smaller in amplitude and faster (taking 1 sec to cross the egg) than the activating wave. The last row of images shows the first of the stably localized calcium waves in this particular egg. **(b)** The full establishment of the cortically propagating calcium waves in an egg that was in mid-meiosis (beginning around 600 sec after extrusion of the first polar body). The three rows of images are taken from the same egg. The first calcium wave begins to propagate cortically and originates from the now relaxed contractile region (marked c) opposite the polar body (pb). All 4/4 eggs analyzed had late post-activation calcium waves that propagated cortically (18–21 $^{\circ}\text{C}$).

Return to text reference [1] [2] [3] [4] [5] [6] [7]

As meiosis continued, however, the later, post-activation calcium waves began to propagate preferentially in the egg cortex, and the wavefront is convex by the time it reaches the anti-pole from its point of origin (Fig. 4b). At around this time — about 5–10 minutes after fertilization — distinct domains were easily distinguishable within the cytoplasm. These ER-rich and ER-poor domains (5–10 μm diameter) are responsible for the characteristic granular appearance of eggs midway through meiosis [21].

Late periodic post-activation calcium waves take longer to cross the egg due to their cortical path

For technical reasons, we used low-light-level video microscopy to record the activation calcium wave and a full series of post-activation calcium waves. We observed that the first few periodic post-activation calcium waves were faster than the activation wave (see the waves labelled 1–3 in Fig. 2), whereas the later waves slowed down (see waves labelled 4–6 in Fig. 2). Table 1 shows the details of the wave-speeds. Having performed confocal microscopy, it became apparent that the late slowing of the post-activation calcium waves (as shown in Fig. 2) was due to an increasing tendency for the waves to propagate predominately in the egg cortex. Ratios of confocal images of the activation wave and two post-activation calcium waves (Fig. 4a) show that the second post-activation calcium wave begins to display a tendency to propagate more cortically. This tendency towards a cortical propagation increases 5–10 minutes post-fertilization coinciding with the development of the ER-rich and ER-poor domains. All post-activation calcium waves occurring between 10 and 20 minutes after fertilization — the time between extrusion of the first and second polar bodies — propagate cortically. Fig. 4b shows three examples of these cortical calcium waves. All these post-activation calcium waves initiate from the relaxed contractile pole opposite the polar body (labelled pb in Fig. 4b). Again, these waves also cross the egg more slowly than the first post-activation calcium waves, but only because they follow a longer path around the cortex. If we assume an exclusively cortical path, the wave-speed would be increased by ~50%. Thus, as seen in Fig. 2 and Table 1, the slower wave-speeds of the late versus the early post-activation calcium waves — here considered as the time taken to cross the egg — probably reflect the fact that the late post-activation calcium waves propagate cortically.

Table 1. The wave-speed first increases following activation (after approximately 5 minutes then apparently slows down again towards the end of meiosis.

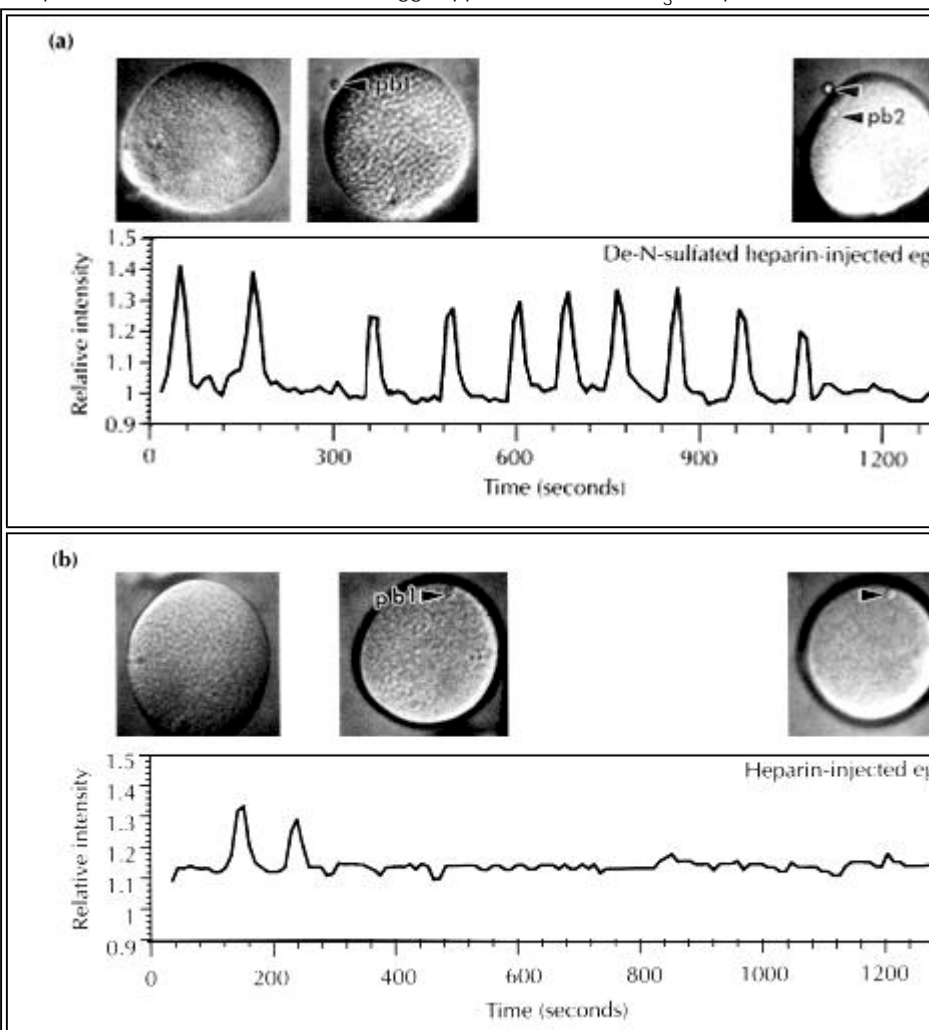
Activation wave-speed ($\mu\text{m s}^{-1}$)	Early post-activation wave-speed ($\mu\text{m s}^{-1}$)	Late post-activation wave-speed ($\mu\text{m s}^{-1}$)
5 ± 1	9 ± 1	6 ± 1.5
(mean \pm SD, $n = 9$)	(mean \pm SD, $n = 9$)	(mean \pm SD, $n = 9$)

These data were collected using low-light-level video microscopy. Because the egg deformation distance travelled by each wave was slightly different and was therefore determined for each individual egg and the speed calculated. The post-activation calcium waves are significantly faster than the activation wave ($p = 0.0004$, Student's two-tailed paired t-test). However, if we assume that the late post-activation waves propagate cortically, the distance travelled increases by over 50 %, which gives a wavespeed of approximately $9 \mu\text{m s}^{-1}$, essentially identical to the early post-activation calcium waves.

Return to table reference [1] [2] [3]

Periodic post-activation calcium waves are necessary for the completion of meiosis II

To determine whether the post-activation calcium waves are necessary for the completion of meiosis, we attempted to interfere with the calcium signals by injecting fertilized eggs with a competitive inhibitor of InsP_3 -induced calcium release [34] [37]. As the concentrations of InsP_3 used may have other effects, we injected de-N-sulfated heparin, which does not inhibit InsP_3 -induced calcium release, as a control (see Fig. 5a). De-N-sulfated heparin did not inhibit the activation calcium waves, polar body extrusion or cleavage (Fig. 5a, 8 / 8 eggs). Heparin, injected at $250 \mu\text{g ml}^{-1}$ about 1 minute after the activating wave, inhibited most of the post-activation waves; heparin also prevented extrusion of the second polar body (Fig. 5b, 8 / 8 eggs). Neither ryanodine nor cyclic ADP-ribose — the putative endogenous messenger for the non-skeletal ryanodine receptor [38] — elicited a $[\text{Ca}^{2+}]_i$ increase (data not shown), so the only relevant calcium channels in ascidian eggs appear to be the InsP_3 receptor channels.



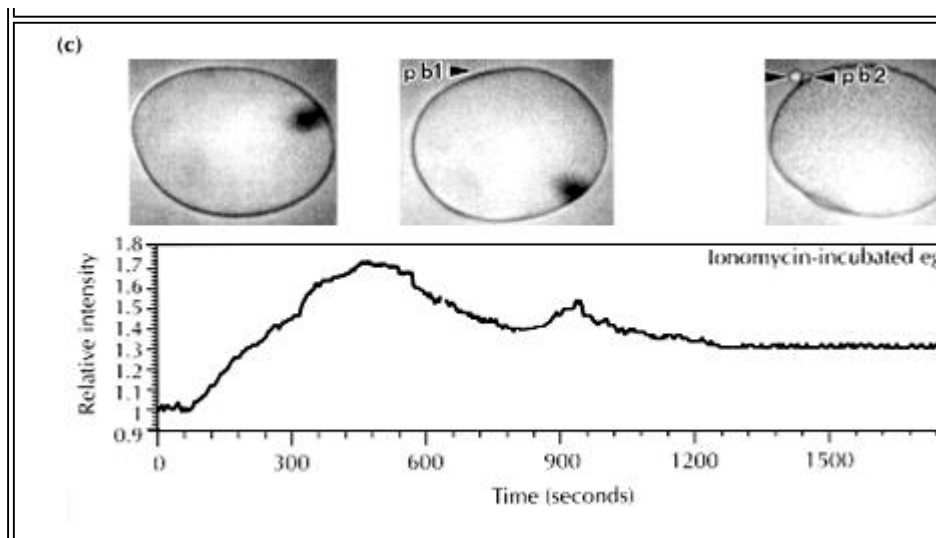


Fig. 5. The role of calcium in the completion of meiosis. Dechorionated oocytes were injected with 10 000 D-dextran-coupled CG ($10\text{--}20\ \mu\text{M}$) and fertilized 30 min later. Images were collected every 10 sec using low-light-level video imaging (see Materials and methods and Fig. 2). Following activation wave, these eggs were injected with either heparin or de-N-sulfated heparin ($250\ \mu\text{g}\ \text{m}^{-1}$). **(a)** The pattern of $[Ca^{2+}]_i$ increases following injection of de-N-sulfated heparin. Here the relative intensity of CG fluorescent emission is plotted against time. Time 0 corresponds to the time of injection (3–5 minutes after fertilization). Those eggs injected with de-N-sulfated heparin had the normal number and pattern of post-activation calcium waves, and completed meiosis giving off two polar bodies (pb1 and pb2); they later divided with the same timing as uninjected eggs ($n = 8$, $18\text{--}21\ ^\circ\text{C}$). **(b)** Heparin was injected ($250\ \mu\text{g}\ \text{m}^{-1}$) just after the activation calcium wave. The images were analysed and the relative intensity of CG emission plotted against time as shown (time 0 is the time of injection). These heparin-injected eggs only had a few post-activation calcium waves (two in this particular egg) after the injection; the others were suppressed. These eggs did not complete meiosis II (see bright-field images showing only one polar body, pb1) and did not divide ($n = 8$, $18\text{--}21\ ^\circ\text{C}$). **(c)** The pattern of the $[Ca^{2+}]_i$ increases in an ionomycin-injected egg ($10\ \mu\text{M}_i$). The relative pixel intensity is plotted against time. This ionophore results in a persistent and homogeneous increase in internal calcium, which induces the extrusion of two polar bodies on time (pb1 and pb2) ($n = 10$, $18\text{--}21\ ^\circ\text{C}$).

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The importance of post-activation $[Ca^{2+}]_i$ increases for the completion of meiosis was also demonstrated in experiments using caged InsP_3 , which allows InsP_3 to be released within the egg by ultraviolet photolysis. One large pulse of UV light generated enough InsP_3 to cause the egg to undergo cortical contraction and emit one polar body. The completion of meiosis II, and the emission of the second polar body, required additional UV pulses, to release InsP_3 and generate additional $[Ca^{2+}]_i$ increases after emission of the first polar body (data not shown). These observations strongly suggest that completion of meiosis II is dependent on further $[Ca^{2+}]_i$ increases following the activation-induced increase.

Although they represent the natural stimulus, periodic $[Ca^{2+}]_i$ increases are not strictly necessary by themselves. The calcium ionophore A23187 was found to activate eggs of the ascidian *C. intestinalis*, which underwent abortive divisions [39] and emitted both polar bodies [40]. We also found that incubation of *P. mammillata* eggs with the calcium ionophore ionomycin, which induces a persistent $[Ca^{2+}]_i$ increase, leads to extrusion of the two polar bodies on time (E

Discussion

Progressive localization of early post-activation calcium wave

About 40 seconds after fertilization of an ascidian egg and the accompanying calcium wave vegetally directed cortical contraction sweeps the incorporated sperm nucleus and both cortical sub-cortical organelles towards a vegetal contraction pole [6] [21]. As the internal calcium returns towards resting levels — 2–3 minutes after fertilization — the first post-activation calcium wave is triggered. The cortical ER, the incorporated sperm, and the origin of the post-activation calcium waves all move towards a vegetal contraction pole. The first post-activation calcium waves show progressive localization of their origin, which normally occurs via the shortest route around the cortex on the side that the sperm entered the egg.

As the localization of the calcium waves is slower than that of surface-attached particles, th

pacemaker is probably not located directly at the cell surface or the plasma membrane. It is likely that the incorporated sperm may sensitize the surrounding cytoplasm through proper introduction of a calcium-release factor, as suggested for other species [12] [41]. Indeed, injection of a bacterial cytoplasmic sperm factor has been shown to induce repetitive calcium spikes in hamster egg. Another possibility is that the cortical contraction causes a local accumulation of cortical organelles such as the ER, which move towards the vegetal contraction pole, but the movement of organelles is slower than that of the surface-attached particles.

An ER-rich domain in the contraction pole becomes the pacemaker of latter calcium waves

Our findings confirm and extend the results of Speksnijder [16], who provided experimental evidence that the pacemaker at the contraction pole is probably the ER domain situated there. We showed that displacing the mitochondria-rich domain by centrifugation displaced neither the pacemaker nor, more importantly, the origin of the post-activation calcium waves [16]. Although Speksnijder's data clearly suggested that the ER domain at the contraction pole could be the pacemaker, one technical drawback was that the spatial resolution of the aequorin signal was limiting.

To reach the necessary spatial resolution, we have used a confocal microscope and two decoupled fluorescent dyes: the calcium-sensitive dye, calcium green-1, and the calcium-insensitive dye, Texas Red. Our experiments show that the origin of the calcium waves occurs in the contraction pole (see Fig. 3). As the temporal resolution was limiting when using the full confocal section, we scanned a single line through the region of origin of the repetitive calcium waves. These experiments show that the $[Ca^{2+}]_i$ increases first in the contraction pole, a micron or two below the plasma membrane. Our observations imply that the origin of this periodic release of calcium is the domain of ER tubes and sheets that is highly concentrated in the contraction pole beneath the plasma membrane, rather than the mitochondria-rich domain (the myoplasm) that is situated further away from the plasma membrane [21].

We do not know the mechanism by which the periodic calcium waves are generated, but we know that calcium influx is not involved, as external calcium in the sea water is not required to support the periodic $[Ca^{2+}]_i$ increases [5]. Experimental evidence from isolated rat and mouse pancreatic acinar cells shows that calcium waves propagate from a trigger zone in the secretion pole, a specialized cytoplasmic region that seems to contain sensitized $InsP_3$ receptors [42]. Likewise, there may be either accumulated or sensitized $InsP_3$ receptor channels in the ascidian contraction pole that may be triggered to open as the luminal calcium pumped inside the ER reaches a critical level. Such a scenario is supported by experimental evidence which shows that increasing luminal calcium can stimulate the $InsP_3$ receptor channel to open [44] [45].

At this point we can only speculate about the source of the variations in the speed of propagation of the calcium waves already noted [14]. It is likely that the smaller amplitude of the post-activation calcium waves (as compared to the activation wave) and their increased speed are related. For example, there is a surge in the $InsP_3$ level following fertilization in the ascidian egg, as in the sea urchin and *Xenopus* eggs [8] [46], and if the $InsP_3$ receptor channel is sensitized by the increase in $InsP_3$, the post-activation calcium waves could propagate at a faster rate and larger amplitude. For this to occur we must assume that calcium is the diffusible messenger and that an increase in cytoplasmic calcium induces the $InsP_3$ receptor channel to open. Lechleiter and [47] have tested the temperature-dependence of wave-front propagation in *Xenopus* oocytes and demonstrated that calcium does seem to be the diffusible messenger, and in planar lipid bilayers containing channels from the ER of canine cerebellum cells, the probability of the $InsP_3$ receptor channel being open displays a bell-shaped response to physiological $[Ca^{2+}]_i$ increases [48].

Cortically propagating calcium waves

Analysis of the speed of waves visualized with low-light-level microscopy (see Table 1) shows that the early post-activation calcium waves are faster (9 versus $5 \mu m s^{-1}$) than the activating waves. However, later post-activation calcium waves slow down again to around $6 \mu m s^{-1}$. This slowing could be due to their cortical propagation, which would effectively increase the distance covered by the waves by up to 50%. The actual rate, if one assumes that the wave propagates only at the egg cortex, is faster than the activation wave and approaches the rate of the early post-activation waves.

It is interesting to note that a change in the local cytoplasmic geometry accompanies the cortical propagation phenomena; ER-rich domains start forming, separated by yolk-granular domains, after the emission of the first polar body. The domains increase in size giving the granular appearance [18] [21]. These ER-rich domains in the centre of the egg become about $1 \mu m$ in diameter and are separated by equally large yolk-rich domains; positive feedback through the diffusion of calcium may be hindered in such an environment where the distance between $InsP_3$ receptors in the ER tubes and sheets may effectively increase. In fact, it has been shown that

propagating calcium waves rely on several factors, which include local geometry and buffer characteristics, as well as channel and pump behaviour (see [49] for a discussion).

A possible function for the periodic post-activation calcium waves in ascidian and mammalian eggs

In many vertebrate oocytes, cell-cycle arrest occurs at the metaphase–anaphase transition second meiotic division. In *Xenopus*, the calcium-dependent enzyme CaMKII is probably responsible for the destruction of cyclin, exit from M phase and the subsequent loss of the cytostatic factor [25]. A role for calcium in exit from M phase is supported by data from mouse oocytes, showing completion of meiosis may be triggered by a transient increase in cytosolic calcium ions. The blocking of the calcium increases in fertilized mouse eggs with BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) inhibits second polar body formation [50], and cell-cycle progression of parthenogenetically activated mouse oocytes is dependent on the $[Ca^{2+}]_i$ [5]. In addition, the presence of an intact spindle at the time the $[Ca^{2+}]_i$ increases appears necessary for completion of the second meiosis [23]. In contrast to the vertebrate situation, the metaphase block of the gastropod mollusc *Patella*, the bivalves *Mytilus* and *Ruditapes*, and the ascidian *mammillata* may be relieved at activation by CaMKIII, which phosphorylates and inhibits EF2, hence causing a drop in the level of protein synthesis [52]. The activation wave may therefore be due to the loss of some short-lived protein(s) which relieves the cell-cycle arrest and permits the ascidian egg to complete meiosis I.

Here, we have concentrated on determining which calcium signals are required for the completion of the meiotic divisions. We have shown that injection of a competitive inhibitor of $InsP_3$ -induced calcium release, heparin [34] [37], into fertilized ascidian eggs between fertilization and exit from the first polar body, inhibits the post-activation calcium waves and blocks completion of meiosis II. These experiments, and those using caged $InsP_3$, show that the activation calcium wave is not enough of a stimulus to induce completion of meiosis I, but that additional calcium signals are required for completion of meiosis II.

Histone H1 kinase activity falls after the activation calcium wave and increases again after exit from the first polar body, during the time that the internal calcium periodically increases (A.M. unpublished observations). These preliminary results imply that, in the ascidian egg, an increase in histone H1 activity accompanies increases in cytoplasmic calcium levels, contrary to the suggestion that calcium signals inactivate MPF by inducing the destruction of cyclin [25]. It will be interesting to determine whether the histone H1 kinase activity stays high in ascidian eggs that have had post-activation calcium waves inhibited. If the histone H1 kinase activity does stay high, it may account for the observation that the second polar body is never extruded, and would then be in agreement with the suggestion that calcium increases can inactivate MPF. Thus, in the fertilized ascidian egg, the post-activation calcium increases may eventually lead to destruction of MPF.

It is clear that there are ways to induce the completion of meiosis II other than exposing the egg to periodic $[Ca^{2+}]_i$ increases, as a calcium ionophore can induce completion of meiosis by inducing a persistent $[Ca^{2+}]_i$ increase (see Fig. 5c). In this case, however, the eggs do not progress to gastrulation. It is possible that periodic calcium waves are essential for later developmental events suggested for mouse eggs [33]. In this regard, we should point out that in ascidians it has been shown that if the vegetal pole — the region that contains the calcium wave pacemaker — is removed, the onset of gastrulation is prevented [53].

Conclusions

Our observations clearly show that a persistent or periodic rise in $[Ca^{2+}]_i$ is required for the completion of meiosis I and II in the ascidian egg. This effect could be mediated by the activation of CaMKII, destruction of cyclin and inactivation of MPF. We have observed that the origin of periodic calcium waves in the ascidian egg gradually localizes to a dense accumulation of ER at the vegetal pole, which acts as a pacemaker. As the contraction pole becomes the future site of gastrulation in the embryo, the periodic calcium waves that originate there may have developmental significance.

We suggest that the generation of periodic calcium waves could be a result of sensitization to the accumulation of $InsP_3$ receptors in this ER-rich region. The post-activation calcium waves may speed up and to propagate cortically around the time that the first meiotic cycle is complete. The cortical path may reflect the documented changes in the structure of the egg cytoplasm (see Fig. 5). In particular, ER-rich domains that form in the center of the egg may become too distant to provide positive feedback through calcium-induced calcium release acting on $InsP_3$ receptor channels. These features of the fertilized ascidian egg make it an excellent model system for studying the role of repetitive calcium signals as well as the mechanisms involved in the generation and propagation of calcium waves.

Materials and methods

Biological material and chemicals

We usually collected the European ascidian *Phallusia mammillata* at Sète (Mediterranean coast) occasionally from Roscoff (Brittany coast). The animals were kept in the aquaria in the Mari Biology Station at Villefranche sur Mer at 16–22 °C. Denuded oocytes were handled and described previously [18]. To obtain the gametes, the animals were first removed from the and the gametes collected. Sperm were kept at 4 °C and as dry as possible. Oocytes were TAPS (N-tris[hydroxymethyl]methyl-3-aminopropane-sulphonic acid)-buffered sea water (8.2) at 16–22 °C. The chorions were subsequently removed from the oocytes by treatment % trypsin (Sigma Chemical Company) in TAPS-buffered sea water at around 18–21 °C for : Dechorionated oocytes were kept in 0.1 % gelatin-formaldehyde coated petri dishes — which prevents sticking and lysis of the nude oocytes — in filtered TAPS-buffered sea water, after washed several times to remove the trypsin [18]. Provided that the denuded eggs and the are left in contact with gelatin-coated surfaces and prevented from sticking to each other, normal tadpoles develop overnight.

Microinjection technique

Injection solution consisted of 180mM KCl, 30mM BES ((N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid), 100 µM EGTA, pH 7.1. Dextran-coupled (10 000 D) calcium green (CG) and Texas Red (TR) (Molecular Probes) were co-injected into eggs to give an internal concentration of around 10–20 µM. We also used a 70 000 D dextran that had both TR and coupled to the same bead in a 1:1 molar ratio (courtesy of Molecular Probes). Heparin and sulfated heparin (Sigma Chemical Company) were injected to give a final intracellular concentration of 250 µg ml⁻¹. Caged InsP₃ (Molecular Probes) was dissolved in 2mM dextran-coupled CG 1mM stock. The resultant solution was later injected giving 10–20 µM CG and 5–10 µM caged InsP₃. Photorelease of the caged InsP₃ was achieved by a 1 sec exposure to UV light (50W mercury lamp) through a 40x/n.a.1.3 oil objective lens, and the increase in calcium was visualized using light-level video microscopy as detailed below.

All surfaces that the oocytes came into contact with were gelatin-coated. Dechorionated oocytes were transferred to small glass wedges mounted onto 400 µl perspex chambers for horizontal microinjection, essentially as described [5] [54]. A high-pressure air microinjection system the desired quantity of injectate (usually between 0.1–0.5 % egg volume or 1.5–7.5 pl), as described [55]. Filamented microinjection electrodes (GC100F-10, Clark Electromedical Instruments) were prepared using a Kopf vertical puller (model 720, David Kopf Instruments) injected oocytes were left for 30 min to ensure diffusion of the injectate before fertilization activation.

Imaging intracellular calcium

All experiments were performed at 18–21 °C. Preactivated sperm (incubated for 30 min with dechorionated oocytes) were delivered to the preparation to fertilize the oocytes [18]. Eggs microinjected with dextran-coupled CG and TR (in their microinjection chamber) were mounted on the stage of a Leica confocal microscope equipped with an argon-krypton laser. CG and TR were excited at 488 and 568 nm, respectively. The optical section was chosen to be 1–2 µm thick. The emitted fluorescence was separated by a 580 nm dichroic filter and collected simultaneously on two separate photomultipliers, using a 532 nm band-pass filter for the CG fluorescence and a 590 nm long-pass filter for the TR fluorescence. Images were collected every 2 sec and digitized in 256 format. We also performed line-scans through the contraction pole. We averaged 32 line-scans to produce a 256 × 256 confocal image. Ratio data (CG/TR) was obtained by performing a pixel-by-pixel division. Emitted fluorescence was collected using a 40x/n.a.1.3 oil objective lens using an upright Leica microscope.

To permit low-light-level video microscopy, the injection chamber was mounted onto the stage of an upright Zeiss Axiophot microscope equipped with epifluorescence, computer-controlled camera, a shutter and recorder using a fluorescein filter set (excitation filter 450–490 nm band-pass, dichroic beamsplitter 510 nm, emission filter 520 nm long-pass) and a 50W mercury vapour lamp. The emitted CG-dextran light was collected using a 40x/n.a.1.3 oil objective lens and a SIT (Silicon Intensified Target) camera (Lhesa, Paris, France) and digitized (through a Matrox card) using software loaded with the Image 1 software (Universal Imaging). The digitized images were stored on an optical disc recorder (OMDR, Panasonic) at a rate of 15 images per min and analyzed subsequently with the image 1 software.

To quantify the [Ca²⁺]_i, we calibrated our system using previously determined values for [Ca²⁺]_i. We fertilized injected sea urchin and ascidian eggs to obtain a relative CG pixel intensity — [Ca²⁺]_i in both species is known, from fura-2 and aequorin data [5] [30] [31], to rise to ~2.33 from a resting level of ~100 nM on fertilization, and we recorded a mean relative change in CG pixel intensity of 2.33 (n = 3) during the fertilization-induced [Ca²⁺]_i increase. Using these values to calculate an apparent dissociation constant (K_{app}), and values for I_{sat} and I_{min} —

by $I_{\text{sat}}/I_{\text{min}} = 14.0$, saturating to minimal fluorescence (Molecular Probes and [22]) — we v to estimate the internal calcium concentration from the equation $[Ca^{2+}]_i = K_{\text{app}}(I - I_{\text{min}})/(I_{\text{sa}}$ problem we had, however, was that CG did not seem to report the high $[Ca^{2+}]_i$ increases (measured with aequorin [5]) characteristic of the activation wave in *P. mammillata*, probat because the fluorescent dye saturates with high concentrations of calcium.

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