

### Function and characteristics of repetitive calcium waves associated with meiosis

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### Abstract

### Backgound

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<sup>2+</sup>], increases are in t of waves, the origin of which relocalizes to a pacemaker region, and that they stop seconds the completion of meiosis.

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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 r that takes about five minutes. Once the origin of these repetitive post-activation calcium w reached the contraction pole, all subsequent calcium waves originate from the domain of EF 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-ac calcium waves begin to propagate preferentially in the egg cortex. By manipulating intracel calcium levels with caged inositol 1, 4, 5 trisphosphate (InsP<sub>3</sub>) and a competitive inhibitor ( induced calcium release, we show that the activation wave induced by the sperm is sufficien induce extrusion of the first polar body, but that additional [Ca<sup>2+</sup>], increases are necessary completion of the second meiotic division. However, periodic calcium waves per se do not s be strictly necessary for the completion of meiosis, as a persistent and homogeneous increa calcium, induced by the calcium ionophore ionomycin, is sufficient to cause second polar bc formation and allow completion of meiosis on time.

### Conclusion

These results clearly show that, in the ascidian egg, post-activation calcium waves are required 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 fill becomes a pacemaker from which the calcium waves originate. Once their origin becomes s localized, the calcium waves begin to propagate preferentially around the cortex of the egg than throughout the egg cytoplasm.

### Background

Calcium waves are a central feature of fertilization in many deuterostome eggs [1] and, in 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-tri increase in the level of free internal calcium  $([Ca^{2+}]_i)$  [1] [5] [6] [7] [8] [9]. Although the of many deuterostome eggs stimulates just a single calcium wave [8] [9] [10], in mammal ascidian eggs, fertilization has been shown to be followed by a series of repetitive  $[Ca^{2+}]_i$  i in addition to the activation wave [5] [11] [12] [13].

In contrast to mammalian eggs, where most periodic [Ca<sup>2+</sup>], increases appear synchronous

throughout the egg cytoplasm [13], the periodic  $[Ca^{2+}]_i$  increases in eggs of ascidians (suc *Phallusia mammillata*) occur in the form of waves [14]. The first, and activating, calcium wat riggered preferentially in the animal hemisphere by the sperm that enters the egg there. F 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 activat calcium wave presumably induces the contraction of a cortical actomyosin basket (open arc animal pole region), thereby stimulating the first phase of ooplasmic segregation [6] [17] [Besides concentrating a mitochondria-rich domain (the myoplasm) in the vegetal hemisphe [19], this contraction also induces an endoplasmic reticulum (ER) -rich domain to form in the misphere at a 'contraction pole' [20] [21]. Although, in these experiments, the lateral re obtained by photon imaging of aequorin luminescence was limited (to around 10–20 µm<sup>2</sup>), observed stratification of mitochondria away from the contraction pole strongly suggested t 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 en 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, a cytoplasmic space varies in the different domains. To overcome this dye-distribution proble have simultaneously injected two 10 000 D molecular weight, dextran-coupled fluorescent 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- $\epsilon$ we determine the ration of simultaneously acquired confocal images in real time. This appre removes potential artifacts, including dye internalization and dye-buffering of internal calciu use relatively low intracellular concentrations of CG) [22]. By using confocal microscopy, w been able to improve our spatial resolution to around 2–5 µm in the *x*–*y* plane and 1–2 µm plane, allowing precise characterization of the spatial aspects of these periodic calcium wav

It is not clear what role the nost-fartilization calcium signals have on meiosis hut it has her

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 b brought about by a drop in the level of cyclin B, and consequent inactivation of M-phase profactor (MPF) [23] [24]. In *Xenopus* eggs, the calcium-induced activation of calmodulin kina (CaMKII) induces the requisite cyclin destruction [25]. Indeed, if constitutively active CaMk overexpressed in the yeast *Schizosaccharomyces pombe*, the p34<sup>cdc2</sup> 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-de oscillations in the InsP<sub>3</sub> level [32]. These data suggest that the cyclin destruction machiner

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, arti 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 con meiosis after fertilization; during this time, a series of calcium waves crosses the egg, and second polar body is immediately extruded when the calcium waves stop [5] [14]. This ten correlation has led us to suggest that these periodic calcium waves play a role in the compl meiosis II [5] [14]. We have now examined this possibility by inhibiting these calcium wave known InsP<sub>3</sub> receptor channel-blocker [34], and inducing meiosis by artificially raising the | eggs of the ascidian *P. mammillata*.

### Results

### I maging free intracellular calcium in ascidian eggs

We imaged calcium in ascidian eggs using the fluorescent calcium dye CG coupled to dextra 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 The activating calcium wave passed through the centre of the egg and propagated essentia constant speed throughout the cytoplasm. Fig. 1b shows the pixel intensity of the experime displayed in Fig. 1a. This indicates that, under our experimental conditions, the calcium-ser CG signal can be isolated from the calcium-insensitive TR signal. We can therefore determin CG/TR ratio, which gives us a good quantitative estimate of the internal calcium concentrat sufficient spatial resolution.





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 completing meiosis and extruding the second polar body. In the fertilized ascidian egg, ther distinct domains of cytoplasm in which the composition of organelles varies; these domains varying amounts of the dextran-coupled dyes. If we used only CG, the bright areas could b misinterpreted as regions where the resting internal calcium concentration is higher. As the 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. I image in Fig. 1c shows that this is the case. This not only confirms that the dyes are concer certain cytoplasmic domains, but it is also good indirect evidence that, after microinjection, 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 the CG and TR dyes coupled to it (with a 1:1 molar ratio of both dyes per dextran). To estir resolution in the x-y plane, we injected eggs before fertilization and imaged the resting intercalcium level in the polar body (which is ~ 4 µm in diameter). In some cases, the polar boc internal calcium level oscillated out of phase with the egg. This allowed us to determine if w resolve  $ICa^{2+1}$  increases in small cytoplasmic areas using our ratiometric methodology. As

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able to detect  $[Ca^{2+}]_i$  increases within the polar body which persisted in the ratiometric imation  $\underline{Fig. 1d}$ , 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 orig 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 under our experimental conditions. We could detect  $[Ca^{2+}]_i$  increases as the tadpole beat i = 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 cro egg as a wave from the site of sperm–egg fusion ([14] [35] and Fig. 1a). Using aequorin, been shown that the peak  $[Ca^{2+}]_i$  increase at activation is ~ 7 µM, whereas the later postcalcium waves are 1–4 µM [5]. We have confirmed that all the post-activation  $[Ca^{2+}]_i$  increase 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 wit fluorescent dyes has allowed us to show that the origin of the post-activation calcium wave indeed move 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 loc 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 local waves (see waves marked 1–3).

act (0s) 1 (128s) 2 (160s) 3 (220s) 4 (264s) 5 (328s) 6 (436s) Act (0s) Act (0

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

linjected with 10 000 D-dextran-coupled CG (10–20  $\mu$ M) and fertilized 30 min later. Image collected at 4 sec intervals using a SIT camera, the Image 1 software and an OMDR to sto images. The images are displayed to indicate  $[Ca^{2+}]_i$  increases as clearly as possible — by subtracting previous images (n–(n–1)), the sites of initiation (arrowheads) of the waves an direction of propagation (arrows) are visualized most clearly. The first seven calcium wave the same egg are shown, beginning with the first, activating wave (the row of images mar 'act') that originates from the site of fertilization (arrowhead f). The periodic calcium wave follow are numbered 1–6, and their sites of origin are indicated (arrowheads), as are the t which the waves begin. The site of origin becomes progressively localized following fertilization cocurs) to the contraction pole (labelled c) and the direction of propagation (ar 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 rn preferentially cortical propagation (see text and Fig.4). All subsequent calcium waves com 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 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 part 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 c wave right under a particle (near, but not exactly on, the animal pole). In this case we clea that the surface particle was swept towards the vegetal hemisphere and the contraction pol before the calcium waves localized to the same region (data not shown). This demonstrates 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 req contraction pole (marked c in Fig. 2, wave 4). The contraction pole is a cell-surface protrus situated within 50 ° of the vegetal pole, and is the result of a cortical contraction triggered activation wave [6]. We previously suggested that the late, stable pacemaker consists of at domain situated in the contraction pole [14] [20] [21], and experiments based on the reloc of cytoplasmic domains make this a very likely possibility [16].

### The ER-rich domain in the contraction pole becomes the calciu 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 dextra the egg and subsequently imaged ~ 1 µm-thick, equatorial confocal sections in the contrac 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 (markec second row of images in Fig. 3a) just as the calcium waves first became stably localized (~ minutes after fertilization). In this figure, ratio images (CG/TR) are shown together with TR these reveal the presence of distinct cytoplasmic domains in the confocal sections. In order identify the onset of the periodic post-activation calcium waves, we recorded several succe: waves at high magnification, while precisely adjusting the level of the section to localize the of the pacemaker. We scanned every 2 seconds, which gave a good compromise between t 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 usir confocal images of three successive calcium waves that come from the same area in the c 18-21 °C). The five ratio images in each scene are shown, together with a TR image (pan showing the cytoplasmic domains in that region (m, myoplasm or mitochondria-rich doma using a zoom function, the area from which the periodic waves come has been expanded ( analysis indicates that the [Ca<sup>2+</sup>], increase first occurs (arrowhead) in the contraction pole plasma membrane (arrowhead). The  $[Ca^{2+}]_i$  increase begins at the level of the plasma me domain of concentrated ER resides (n = 6, 21 °C). Note also the overall lower  $[Ca^{2+}]_i$  incr 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 confc through the contraction pole (marked c), as illustrated in the TR image on the extreme rig × 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 descendir vertical axis in this panel. This further improves both the lateral resolution and the tempor 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. display the pixel intensity in that area) showed that the  $[Ca^{2+}]_i$  increases first in the contrapole 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 measing further by scanning a single line, passing through the site of origin of the later post-activat calcium waves, that began in the contraction pole. The TR, CG and ratio (CG/TR) images ar 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 t domain is located. These experiments show that the cortical ER domain at the contraction p 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 esse 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 spl 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 v 000 D-dextran-coupled CG and TR (10–20  $\mu$ M) and fertilized 30 min later. Ratio images (C were collected every 2 sec using CLSM. **(a)** The activation calcium wave (act) travels thro centre of the egg from the site of sperm–egg fusion (arrowhead), crossing it in 24 sec. The row of images shows the next wave in this egg, which is a progressively localizing one; thi also travels through the centre of the egg, but is smaller in amplitude and faster (taking 1 cross the egg) than the activating wave. The last row of images shows the first of the stab 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 a extrusion of the first polar body). The three rows of images are taken from the same egg. calcium wave begins to propagate cortically and originates from the now relaxed contractic (marked c) opposite the polar body (pb). All 4/4 eggs analyzed had late post-activation ca waves that propagated cortically (18–21 °C).

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

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

For technical reasons, we used low-light-level video microscopy to record the activation cal wave and a full series of post-activation calcium waves. We observed that the first few peri 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). Ta shows the details of the wave-speeds. Having performed confocal microscopy, it became at that the late slowing of the post-activation calcium waves (as shown in Fig. 2) was due to a increasing tendency for the waves to propagate predominately in the egg cortex. Ratiometr confocal images of the activation wave and two post-activation calcium waves (Fig. 4a) shc the second post-activation calcium wave begins to display a tendency to propagate more cc This tendency towards a cortical propagation increases 5–10 minutes post-fertilization conc with the development of the ER-rich and ER-poor domains. All post-activation calcium wave occurring between 10 and 20 minutes after fertilization - the time between extrusion of th and second polar bodies - propagate cortically. Fig. 4b shows three examples of these calc waves. All these post-activation calcium waves initiate from the relaxed contraction pole op the polar body (labelled pb in Fig. 4b). Again, these waves also cross the egg more slowly t first post-activation calcium waves, but only because they follow a longer path around the c we assume an exclusively cortical path, the wave-speed would be increased by ~50 %. The as seen in Fig. 2 and Table 1, the slower wave-speeds of the late versus the early post-acti calcium waves - here considered as the time taken to cross the egg - probably reflect the the late post-activation calcium waves propagate cortically.

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

Activation wave-speed (µm s <sup>-1</sup> )	Early post-activation wave- speed (µm s <sup>-1</sup> )	Late post-activation wa speed (µm s <sup>-1</sup> )	
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 deform distance travelled by each wave was slightly different and was therefore determined for ea individual egg and the speed calculated. The post-activation calcium waves are significantl than the activation wave (p = 0.0004, Student's two-tailed paired t-test). However, if we that the late post-activation waves propagate cortically, the distance travelled increases by over 50 %, which gives a wavespeed of approximately 9 µm s<sup>-1</sup>, 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 c 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 used may have other effects, we injected de-N-sulfated heparin, which does not inhibit Insl induced calcium release, as a control (see Fig. 5a). De-N-sulfated heparin did not inhibit th activation calcium waves, polar body extrusion or cleavage (Fig. 5a, 8 /8 eggs). Heparin, inj 250 µg ml<sup>-1</sup> 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). Neit ryanodine nor cyclic ADP-ribose — the putative endogenous messenger for the non-skeleta ryanodine receptor [38] — elicited a  $[Ca^{2+}]_i$  increase (data not shown), so the only relevar receptor calcium channels in ascidian eggs appear to be the InsP<sub>3</sub> receptor channels.





Fig. 5. The role of calcium in the completion of meiosis. Dechorionated oocytes were injec 10 000 D-dextran-coupled CG (10–20 µM) and fertilized 30 min later. Images were collect 10 sec using low-light-level video imaging (see Materials and methods and Fig 2). Followir activation wave, these eggs were injected with either heparin or de -N-sulfated heparin (be 250 μg m<sup>-1</sup>). (a) The pattern of [Ca<sup>2+</sup>]<sub>i</sub> increases following injection of de-N-sulfated hep Here the relative intensity of CG fluorescent emission is plotted against time. Time 0 corre to the time of injection (3-5 minutes after fertilization). Those eggs injected with de-N-su heparin had the normal number and pattern of post-activation calcium waves, and comple meiosis giving off two polar bodies (pb1 and pb2); they later divided with the same timing uninjected eggs (n = 8, 18–21 °C). (b) Heparin was injected (250 µg ml<sup>-1</sup>) just after the activation calcium wave. The images were analysed and the relative intensity of CG emissi plotted against time as shown (time 0 is the time of injection). These heparin injected egg only a few post activation calcium waves (two in this particular egg) after the injection; th ones are suppressed. These eggs did not complete meiosis II (see bright field images show only one polar body, pb1) and did not divide (n = 8, 18–21 °C). (c) The pattern of the [Ca increases in an ionomycin injected egg (10  $\mu$ M<sub>i</sub>). The relative pixel intensity is plotted agai time. This ionophore results in a persistent and homogeneous increase in internal calcium induces the extrusion of two polar bodies on time (pb1 and pb2) (n = 10, 18–21 °C).

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

The importance of post-activation  $[Ca^{2+}]_i$  increases for the completion of meiosis was also experiments using caged  $InsP_3$ , which allows  $InsP_3$  to be released within the egg by ultravial 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 second polar body, required additional UV pulses, to release  $InsP_3$  and generate additional increases after emission of the first polar body (data not shown). These observations strong 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 nec 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]. W also found that incubation of *P. mammillata* eggs with the calcium ionophore ionomycin, wr induces a persistent  $[Ca^{2+}]_i$  increase, leads to extrusion of the two polar bodies on time (F

### 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 corsub-cortical organelles towards a vegetal contraction pole [6] [21]. As the internal calcium towards resting levels -2-3 minutes after fertilization — the first post-activation calcium v triggered. The cortical ER, the incorporated sperm, and the origin of the post-activation cal waves all move towards a vegetal contraction pole. The first post-activation calcium waves progressive localization of their origin, which normally occurs via the shortest route around 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 that the incorporated sperm may sensitize the surrounding cytoplasm through proper introc a calcium-release factor, as suggested for other species [12] [41]. Indeed, injection of a bc cytoplasmic sperm factor has been shown to induce repetitive calcium spikes in hamster eg Another possibility is that the cortical contraction causes a local accumulation of cortical or such as the ER, which move towards the vegetal contraction pole, but the movement of wh 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 experimenta evidence that the pacemaker at the contraction pole is probably the ER domain situated the showed that displacing the mitochondria-rich domain by centrifugation displaced neither the domain nor, more importantly, the origin of the post-activation calcium waves [16]. Althou Speksnijder's data clearly suggested that the ER domain at the contraction pole could be th pacemaker, one technical drawback was that the spatial resolution of the aequorin signal w limiting.

To reach the necessary spatial resolution, we have used a confocal microscope and two dex coupled fluorescent dyes: the calcium-sensitive dye, calcium green-1, and the calcium-inse dye, texas red. Our experiments show that the origin of the calcium waves occurs in the co pole (see Fig. 3). As the temporal resolution was limiting when using the full confocal sectic 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 b the plasma membrane. Our observations imply that the origin of this periodic release of cal the domain of ER tubes and sheets that is highly concentrated in the contraction pole bener plasma membrane, rather than the mitochondria-rich domain (the myoplasm) that is situat 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 require support the periodic  $[Ca^{2+}]_i$  increases [5]. Experimental evidence from isolated rat and mo pancreatic acinar cells shows that calcium waves propagate from a trigger zone in the secre pole, a specialized cytoplasmic region that seems to contain sensitized InsP<sub>3</sub> receptors [42]. Likewise, there may be either accumulated or sensitized InsP<sub>3</sub> receptor channels in the asc contraction pole that may be triggered to open as the luminal calcium pumped inside the EI a critical level. Such a scenario is supported by experimental evidence which shows that inc luminal calcium can stimulate the InsP<sub>3</sub> receptor channel to open [44] [45].

At this point we can only speculate about the source of the variations in the speed of propa the calcium waves already noted [14]. It is likely that the smaller amplitude of the post-act calcium waves (as compared to the activation wave) and their increased speed are related. example, there is a surge in the InsP<sub>3</sub> level following fertilization in the ascidian egg, as the sea urchin and *Xenopus* eggs [8] [46], and if the InsP<sub>3</sub> receptor channel is sensitized by th increase in InsP<sub>3</sub>, the post-activation calcium waves could propagate at a faster rate and re amplitude. For this to occur we must assume that calcium is the diffusible messenger and t increase in cytoplasmic calcium induces the InsP<sub>3</sub> receptor channel to open. Lechleiter and [47] have tested the temperature-dependence of wave-front propagation in *Xenopus* oocyt demonstrated that calcium does seem to be the diffusible messenger, and in planar lipid bil containing channels from the ER of canine cerebellum cells, the probability of the InsP<sub>3</sub> rec channel being open displays a bell-shaped response to physiological  $[Ca<sup>2+</sup>]_i$  increases [48]

#### Cortically propagating calcium waves

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

It is interesting to note that a change in the local cytoplasmic geometry accompanies the or the cortical propagation phenomena; ER-rich domains start forming, separated by yolk-gra 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 abor  $\mu$ m in diameter and are separated by equally large yolk-rich domains; positive feedback thi diffusion of calcium may be hindered in such an environment where the distance between I receptors in the ER tubes and sheets may effectively increase. In fact, it has been shown th 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 refor the destruction of cyclin, exit from M phase and the subsequent loss of the cytostatic far [25]. A role for calcium in exit from M phase is supported by data from mouse oocytes, she completion of meiosis may be triggered by a transient increase in cytosolic calcium ions. The blocking the calcium increases in fertilized mouse eggs with BAPTA (1,2-bis(2-aminopheno. 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]

addition, the presence of an intact spindle at the time the [Ca<sup>2+</sup>]<sub>i</sub> increases appears necess

completion of the second meiosis [23]. In contrast to the vertebrate situation, the metapha block of the gastropod mollusc *Patella*, the bivalves *Mytilus* and *Ruditapes*, and the ascidiar *mammillata* may be relieved at activation by CaMKIII, which phosphorylates and inhibits EF hence causes a drop in the level of protein synthesis [52]. The activation wave may therefc to the loss of some short-lived protein(s) which relieves the cell-cycle arrest and permits th ascidian egg to complete meiosis I.

Here, we have concentrated on determining which calcium signals are required for the com the meiotic divisions. We have shown that injection of a competitive inhibitor of  $InsP_3$ -indu calcium release, heparin [34] [37], into fertilized ascidian eggs between fertilization and ex of the first polar body, inhibits the post-activation calcium waves and blocks completion of II. These experiments, and those using caged  $InsP_3$ , show that the activation calcium wave enough of a stimulus to induce completion of meiosis I, but that additional calcium signals a required for completion of meiosis II.

Histone H1 kinase activity falls after the activation calcium wave and increases again after ( of 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 inc histone H1 activity accompanies increases in cytoplasmic calcium levels, contrary to the su that calcium signals inactivate MPF by inducing the destruction of cyclin [25]. It will be inte 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, th account for the observation that the second polar body is never extruded, and would then t agreement with the suggestion that calcium increases can inactivate MPF. Thus, in the ferti ascidian egg, the post-activation calcium increases may eventually lead to destruction of M

It is clear that there are ways to induce the completion of meiosis II other than exposing th periodic  $[Ca^{2+}]_i$  increases, as a calcium ionophore can induce completion of meiosis by indu

persistent  $[Ca^{2+}]_i$  increase (see Fig. 5c). In this case, however, the eggs do not progress o further. It is possible that periodic calcium waves are essential for later developmental ever suggested for mouse eggs [33]. In this regard, we should point out that in ascidians it has shown that if the vegetal pole — the region that contains the calcium wave pacemaker — is 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 acti CaMKII, destruction of cyclin and inactivation of MPF. We have observed that the origin of  $\mathfrak{g}$  calcium waves in the ascidian egg gradually localizes to a dense accumulation of ER at the contraction pole, which acts as a pacemaker. As the contraction pole becomes the future si gastrulation in the embryo, the periodic calcium waves that originate there may have devel significance.

We suggest that the generation of periodic calcium waves could be a result of sensitization accumulation of InsP<sub>3</sub> receptors in this ER-rich region. The post-activation calcium waves b speed up and to propagate cortically around the time that the first meiotic cycle is complete cortical path may reflect the documented changes in the structure of the egg cytoplasm (se In particular, ER-rich domains that form in the center of the egg may become too distant tc positive feedback through calcium-induced calcium release acting on InsP<sub>3</sub> receptor channe ER. These features of the fertilized ascidian egg make it an excellent model system for stud 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 cc 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 fer 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 ( $\xi$  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 — whi 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,  $\mu$  normal tadpoles develop overnight.

### Microinjection technique

Injection solution consisted of 180mM KCI, 30mM BES ((N,N-bishydroxyethyl)-2aminoethanesulphonic acid), 100  $\mu$ M EGTA, pH 7.1. Dextran-coupled (10 000 D) calcium gr (CG) and texas red (TR) (Molecular Probes) were co-injected into eggs to give an internal concentration of around 10–20  $\mu$ M. 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 conce of 250  $\mu$ g ml<sup>-1</sup>. Caged InsP<sub>3</sub> (Molecular Probes) was dissolved in 2mM dextran-coupled CG 1mM stock. The resultant solution was later injected giving 10–20  $\mu$ M CG and 5–10  $\mu$ M cag Photorelease of the caged InsP<sub>3</sub> was achieved by a 1 sec exposure to UV light (50W mercur lamp) through a 40x/n.a.1.3 oil objective lens, and the increase in calcium was visualized u light-level video microscopy as detailed below.

All surfaces that the oocytes came into contact with were gelatin-coated. Dechorionated oo were transferred to small glass wedges mounted onto 400  $\mu$ l perspex chambers for horizon 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), es as described [55]. Filamented microinjection electrodes (GC100F-10, Clark Electromedical Instruments) were prepared using a Kopf vertical puller (model 720, David Kopf Instrument injected oocytes were left for 30 min to ensure diffusion of the injectate before fertilization activation.

### I maging intracellular calcium

All experiments were performed at 18–21 °C. Preactivated sperm (incubated for 30 min will chorionated oocytes) were delivered to the preparation to fertilize the oocytes [18]. Eggs microinjected with dextran-coupled CG and TR (in their microinjection chamber) were mout the stage of a Leica confocal microscope equipped with an argon-krypton laser. CG and TR excited at 488 and 568 nm, respectively. The optical section was chosen to be 1–2 µm thic emitted fluorescence was separated by a 580 nm dichroic filter and collected simultaneously separate photomultipliers, using a 532 nm band-pass filter for the CG fluorescence and 590 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 in produce a 256 × 256 confocal image. Ratio data (CG/TR) was obtained by performing a sin pixel-by-pixel division. Emitted fluorescence was collected using a 40x/n.a 1.3 oil objective using an upright Leica microscope.

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

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

by  $I_{sat}/I_{min} = 14.0$ , saturating to minimal fluorescence (Molecular Probes and [22]) — we were to estimate the internal calcium concentration from the equation  $[Ca^{2+}]_i = K_{app}(I-I_{min})/(I_{sat})$  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*, probability because the fluorescent dye saturates with high concentrations of calcium.

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