

Three different calcium wave pacemakers in ascidian eggs

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SUMMARY

Calcium wave pacemakers in fertilized eggs of ascidians and mouse are associated with accumulations of cortical endoplasmic reticulum in the vegetal hemisphere. In ascidians, two distinct pacemakers (PM1 and PM2) generate two series of calcium waves necessary to drive meiosis I and II. Pacemaker PM2 is stably localized in a cortical ER accumulation situated in the vegetal contraction pole. We now find that pacemaker PM1 is situated in a cortical ER-rich domain that forms around the sperm aster and moves with it during the calcium-dependant cortical contraction triggered by the fertilizing sperm.

Global elevations of inositol (1,4,5)-trisphosphate (Ins(1,4,5)P₃) levels produced by caged Ins(1,4,5)P₃ or caged glycerol-myoinositol (4,5)P₂ photolysis reveal that the cortex of the animal hemisphere, also rich in ER-clusters, is the cellular region most sensitive to Ins(1,4,5)P₃ and acts as a third type of pacemaker (PM3). Surprisingly, the artificial pacemaker PM3 predominates over the natural

pacemaker PM2, located at the opposite pole. Microtubule depolymerization does not alter the activity nor the location of the three pacemakers. By contrast, blocking the actomyosin driven cortical contraction with cytochalasin B prevents PM1 migration and inhibits PM2 activity. PM3, however, is insensitive to cytochalasin B.

Our experiments suggest that the three distinct calcium wave pacemakers are probably regulated by different spatiotemporal variations in Ins(1,4,5)P₃ concentration. In particular, the activity of the natural calcium wave pacemakers PM1 and PM2 depends on the apposition of a cortical ER-rich domain to a source of Ins(1,4,5)P₃ production in the cortex.

Movies available on-line

Key words: Calcium waves, Fertilization, inositol (1,4,5)-trisphosphate, Cortex, Endoplasmic reticulum, Pacemaker, Eggs, Ascidians

INTRODUCTION

Eggs respond to activating stimuli such as sperm or a variety of parthenogenetic agents by generating calcium waves and in some cases continuing oscillations. Similar waves and oscillations also occur in a variety of somatic cells stimulated by hormones or growth factors (reviewed by Jaffe, 1991; Miyazaki et al., 1993; Whitaker and Swann, 1993; Berridge, 1997). Ongoing repetitive cytosolic [Ca²⁺] ([Ca²⁺]_c) transients following fertilization have been reported in ascidian, mammalian, starfish, mollusc, nemertean and annelid eggs (reviewed by Sardet et al., 1998; Stricker, 1999). While the first calcium wave drives egg activation, the following calcium oscillations are implicated in the completion of meiotic cell cycles and possibly in the proper development of the embryo (reviewed by Jones, 1998; Ozil, 1998).

In eggs, the generation of the meiosis-associated calcium waves is thought to depend mostly on the production of Ins(1,4,5)P₃ triggered by a sperm factor introduced into the egg at fertilization (Jones et al., 2000; Runft and Jaffe, 2000; McDougall et al., 2000; reviewed by Stricker, 1999; Swann and Parrington, 1999). Although the two other calcium-releasing messengers cADPribose and NAADP may mediate a calcium-dependant retrieval of the plasma membrane (Albrieux et al.,

1997; Albrieux et al., 1998), both pathways (NAADP receptor and Ryanodine receptor) do not seem to play a role in the regulation of the sperm-triggered calcium oscillations in the ascidian egg (Albrieux et al., 1997; Albrieux et al., 1998; Yoshida et al., 1998). Ins(1,4,5)P₃ activates Ins(1,4,5)P₃ receptors (Ins(1,4,5)P₃R), located principally in the endoplasmic reticulum (ER) (Miyazaki et al., 1993; Kline et al., 1999; Kline, 2000), by relieving calcium inhibition of the opening of the channel (Mak et al., 1998). A calcium wave mediated by Ins(1,4,5)P₃-induced (calcium-induced) calcium release (ICR) starts with an initial Ca²⁺ increase in a subcellular region that triggers a regenerative propagation of the calcium signal throughout the cell (Berridge, 1997; Marchant and Parker, 2001).

Subcellular regions that repetitively initiate calcium waves (called here 'calcium wave pacemakers') have been described in both somatic cells and zygotes. Why these special regions act as calcium wave pacemakers remains mysterious. In somatic cells, a stable subcellular region located in a fixed position acts as the sole pacemaker site for the initiation of global Ca²⁺ waves triggered by different agonists (Ito et al., 1999; Thomas et al., 1999). In these cells, the distribution of the ER (the main Ins(1,4,5)P₃-sensitive calcium store) appears to underlie the differential sensitivity to Ins(1,4,5)P₃ that

defines the pacemaker site (Lee et al., 1997; Petersen et al., 1999; Thomas et al., 1999). $\text{Ins}(1,4,5)P_3$ diffuses rapidly in the cytoplasm (Albritton et al., 1992) and is thought to act as a global messenger inside the cell (Kasai and Petersen, 1994). However, studies carried out on MDCK cells suggest that $[\text{Ins}(1,4,5)P_3]_c$ waves and oscillations may underlie $[\text{Ca}^{2+}]_c$ oscillations (Hirose et al., 1999). This raises the interesting possibility that complex spatiotemporal patterns of $[\text{Ins}(1,4,5)P_3]_c$ may regulate different calcium wave pacemakers within the same cell.

In both ascidian and mouse zygotes, the meiotic calcium oscillations are all waves that initiate in the cortex and traverse the cell, lasting 20–30 minutes in ascidians (Speksnijder et al., 1989; Speksnijder et al., 1990a; Speksnijder et al., 1990b; Yoshida et al., 1998; Levasseur and McDougall, 2000) and 5–7 hours in mouse (Miyazaki et al., 1993; Deguchi et al., 2000; Jones and Nixon, 2000). In ascidians, sperm-egg fusion triggers two stereotyped series of calcium waves called series I and II (Yoshida et al., 1998). The calcium waves driving meiosis I (series I calcium waves) originate from pacemaker PM1, a sperm-related moving cortical site (McDougall and Sardet, 1995; Roegiers et al., 1995). By contrast, the series II calcium waves, which end at the time of second polar body formation, emanate from pacemaker PM2, which is composed of an accumulation of cortical ER situated in the vegetal cortex of the egg in a constriction called the contraction pole (Speksnijder, 1992; Speksnijder et al., 1993; McDougall and Sardet, 1995; Roegiers et al., 1999). Strikingly, it has been shown recently that in mouse, a vegetal cortical region rich in ER clusters acts as a stable pacemaker of repetitive calcium waves (Kline et al., 1999; Kline, 2000; Deguchi et al., 2000).

One aim of the present work was to examine the structure of the subcellular region in the ascidian zygote cortex associated with the moving pacemaker PM1 by imaging ER-rich domains simultaneously with the site of initiation of calcium waves.

We also wanted to analyse the role of variations in $[\text{Ins}(1,4,5)P_3]_c$ and the importance of cytoskeletal reorganizations in the regulation of the calcium wave pacemaker sites. We used flash photolysis of caged $\text{Ins}(1,4,5)P_3$ (c $\text{Ins}(1,4,5)P_3$) or its poorly metabolized analogue, caged glycerol-myoinositol-phosphatidylinositol (4,5) bisphosphate (cgPtd $\text{Ins}(4,5)P_2$), to raise $[\text{Ins}(1,4,5)P_3]_c$ in the cell and investigated the regional sensitivity of eggs and zygotes to $\text{Ins}(1,4,5)P_3$. Quite unexpectedly, we were able to create a third calcium wave pacemaker (PM3) in the animal pole cortex by globally elevating $[\text{Ins}(1,4,5)P_3]_c$ throughout the cell. Finally we examined the effects of the actin and microtubule depolymerising agents cytochalasin B and nocodazole on the various pacemakers.

Together, our observations suggest that different spatiotemporal patterns of $\text{Ins}(1,4,5)P_3$ concentration and ER distribution underlie the initiation and maintenance of three distinct (one artificial and two physiological) calcium wave pacemakers in the ascidian zygote.

MATERIALS AND METHODS

Biological material

Specimens of the tunicate *Phallusia mammillata* were collected either

from Villefranche sur Mer or Sète (Mediterranean) and kept in sea water at 16–22°C. Denuded oocytes were prepared, handled and fertilized as described previously (Sardet et al., 1989; McDougall and Sardet, 1995).

Microinjection and vital staining

Unfertilized eggs were introduced into a wedge and injected according to the modified method of Hiramoto and Kiehart, as described previously (McDougall and Sardet, 1995). The cytosolic dyes Calcium Green dextran (CG) and Texas Red dextran (TR, 10,000 Da, Molecular Probes), as well as c $\text{Ins}(1,4,5)P_3$ and cgPtd $\text{Ins}(4,5)P_2$ were dissolved in injection buffer (180 mM KCl, 100 μM EGTA, 30 mM BES pH 7.1). Approximately 1% of the egg volume was injected to give a final concentrations of 10–20 μM for CG and TR dextran, 5 μM for c $\text{Ins}(1,4,5)P_3$, and 35 μM for cgPtd $\text{Ins}(4,5)P_2$.

To label the ER network, an oil droplet saturated with DiI $_{(16)}$ (Molecular Probes) was injected into oocytes, essentially as described (Speksnijder et al., 1993). The yolk platelets were labelled by incubating oocytes for 5 minutes in 1 μM of Syto12 (Molecular Probes).

Simultaneous confocal imaging of $[\text{Ca}^{2+}]_c$ and the endoplasmic reticulum

Labelled oocytes were mounted onto the stage of a Leica confocal microscope in a perfusion chamber and imaged (for details, see McDougall and Sardet, 1995). During ratiometric confocal imaging of $[\text{Ca}^{2+}]_c$, each TR image was used to monitor the distribution of ER (see Results; and Figs 2, 3), while CG images were divided by TR images to provide images of the variations of $[\text{Ca}^{2+}]_c$ (McDougall and Sardet, 1995). TR and DiI $_{(16)}$ fluorescence (collected with a 590 nm bandpass filter) were excited by the 568 nm line of the Ar-Kr laser, whereas CG and Syto12 fluorescence (collected with a 530 nm bandpass filter) were excited by the 488 nm line of the laser.

Photorelease of caged compounds and calcium imaging

For UV uncaging of c $\text{Ins}(1,4,5)P_3$ or cgPtd $\text{Ins}(4,5)P_2$ (Bird et al., 1992), oocytes were coinjected with a mixture of CG and TR, and the caged compound. UV-flashes were produced on the Leica confocal microscope with the epifluorescence lamp (75 W mercury lamp). Some uncaging experiments were also performed on an inverted Zeiss microscope (axioplan 100 TV) equipped with a Princeton Instruments frame transfer digital camera, a Ludl stage and filter wheel and an Optiquip 150 W Hg-Xe fluorescent source. From the total number of calcium release events we could trigger in some c $\text{Ins}(1,4,5)P_3$ uncaging experiments, we can estimate the amount of c $\text{Ins}(1,4,5)P_3$ uncaged during each UV-flash. We calculated that the amount of $\text{Ins}(1,4,5)P_3$ generated after each flash represents less than 3% of the injected c $\text{Ins}(1,4,5)P_3$, representing less than 0.1 μM $\text{Ins}(1,4,5)P_3$. Images were acquired and processed using Metamorph 4.0 software (Universal Imaging).

Use of cytoskeletal inhibitors

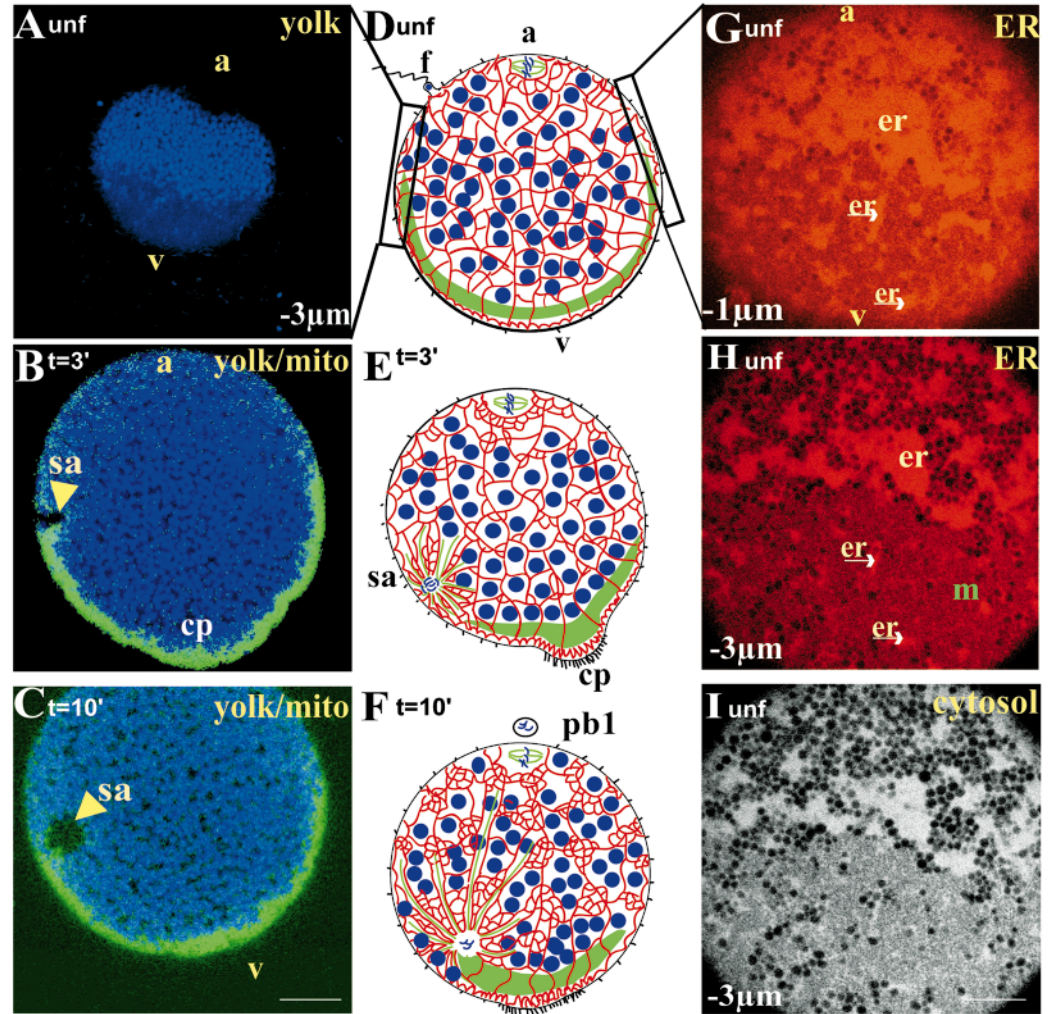
The eggs were held under the microscope in a perfusion chamber and either nocodazole (1.2 μM in sea water, Sigma) or cytochalasin B (1 $\mu\text{g}/\text{ml}$ in sea water, Sigma) perfused. The perfusion lasted less than 1 minute. During that period no recording could be made.

RESULTS

The sperm aster organizes a new peripheral ER-rich domain in the egg within minutes of fertilization

The highly polarized organization of the ascidian egg and zygote (i.e. fertilized egg) is shown in Fig. 1. The drawings in Fig. 1D,E,F represent the cytoplasmic and cortical domains that contain different concentrations of ER, yolk platelets,

Fig. 1. Distribution and reorganization of organelles, cytoplasm and cortex in *Phallusia* eggs and zygotes. Unfertilized egg (A); zygote, 3 minutes after fertilization (B); and zygote, 10 minutes after fertilization (C). The distribution of yolk platelets (blue) and mitochondria (green) in confocal sections 3 μm below the surface (A) or near the equator (B,C) oriented along the animal-vegetal (a-v) axis. The sperm aster region (sa, arrowhead) is a mitochondria-free, yolk platelet-free zone. In B, a contraction pole (cp) has formed. Bar, 23 μm . (D-F) The overall organization of the unfertilized egg (D); zygote, 3 minutes after fertilization (E); and zygote, 10 minutes after fertilization (F). ER network, red; yolk platelets, blue; mitochondria-rich domain, green; microtubules, green lines. Chromosomes (blue) are shown in the sperm aster, in the meiotic spindle and in the polar body (pb1). (G,H) Unfertilized egg: distribution of ER in confocal grazing tangential sections (1 μm (G) or 3 μm (H) below the surface) of an egg oriented along the animal-vegetal (a-v) axis. er, ER-rich domain; er arrow, corridor of ER-rich domain traversing the subcortical ER-poor and mitochondria-rich domain (m in green). (I) Unfertilized egg: distribution of cytosol visualized with injected Ca Green dextran (same egg and confocal section as that seen above in H). Yolk platelets are seen negatively as black round vesicles excluding the dextran dye. Bar, 15 μm .



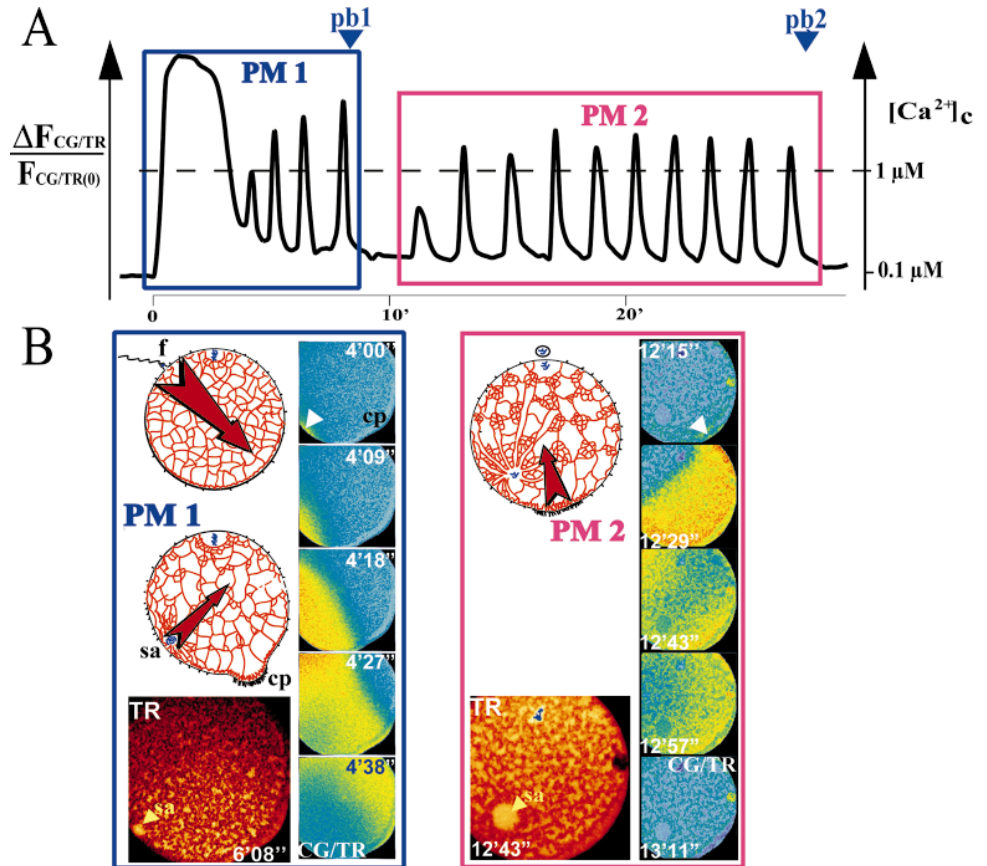
mitochondria and their reorganization after fertilization (Roegiers et al., 1999). Before fertilization, yolk platelets, which are membrane-bound vesicles, are distributed throughout the whole egg but mostly excluded from the cortex by ER-rich domains in the animal hemisphere and the subcortical mitochondria-rich layer in the vegetal hemisphere (Fig. 1A,B). The ER network is made of tubes and sheets and is organized into domains, the size of which increase after fertilization (Speksnijder et al., 1993). In the unfertilized egg, ER-rich domains occupy a large part of the animal cortex and subcortex (Fig. 1G,H, er). However in the vegetal hemisphere, ER-rich domains are restricted to corridors (Fig. 1G,H, arrowed er) traversing a 5–7 μm -thick subcortical mitochondria-rich layer (Fig. 1H, m). Not shown here is the thin and dense monolayer of cortical ER lying against the plasma membrane in the vegetal hemisphere (Sardet et al., 1992; Speksnijder et al., 1993).

Using confocal microscopy, we found that the ER network (labelled with DiIC₁₆₃; Fig. 1G,H) and the cytosol (labelled with the dextran dyes CG or TR; Fig. 1I) have comparable distributions when imaged in the same egg. On average, the ER network occupies about 25% and the cytosol occupies about 75% of the total cytoplasmic space in an ER-rich domain with

no significant contribution from other organelles (EM observations not shown here). Therefore, the visualization of cytosol-rich domains reflects the distribution of ER-rich domains in ascidian eggs and zygotes. As illustrated in Figs 2 and 3, these cytosol-rich/ER-rich domains can be conveniently visualized (TR image) while simultaneously imaging calcium waves (ratio: CG image/TR image).

In ascidians, fertilization is characterized by a spectacular cortical contraction induced by the activating wave of calcium (Brownlee and Dale, 1990; Speksnijder et al., 1990a; Speksnijder et al., 1990b). The wave of cortical contraction starts on the side of the egg where sperm enters, moves vegetally and within 3 minutes concentrates the cortical ER and the subcortical mitochondria-rich domain in the vegetal hemisphere around a constriction called the contraction pole (Fig. 1B,E; Roegiers et al., 1995; Roegiers et al., 1999). We now observe that the sperm aster area can be recognized as a cytosol-rich/ER-rich domain (Fig. 2B, TR, PM1) excluding both yolk platelets and mitochondria (Fig. 1B). Such an ER-rich/yolk platelet-poor region in the centre of the aster can be observed apposed to the cortex towards the end of the cortical contraction, 2–3 minutes after fertilization (Fig. 1B). Soon after the extrusion of the first

Fig. 2. Series I and series II physiological calcium wave pacemakers (PM1 and PM2) are associated with two distinct ER-rich domains. (A) Variations of $[Ca^{2+}]_c$ displayed as normalized ratio CG/TR fluorescence ($\Delta F_{CG/TR}/F_{CG/TR(0)}$) (1 image every 14 seconds). The $[Ca^{2+}]_c$ scale on the right corresponds to earlier quantitative measurements of $[Ca^{2+}]_c$ using aequorin (see Speksnijder et al., 1990a; Speksnijder et al., 1990b; Roegiers et al., 1999). Fertilization elicits series I calcium oscillations (PM1 boxed in blue) and the extrusion of the first polar body (pb1). Series II calcium oscillations (PM2 boxed in pink) can be observed after the extrusion of pb1, in the last 15-20 minutes, and end just before the extrusion of the second polar body (pb2). (B) Only the red lines, which represent the ER network, and the blue chromosomes are depicted. The red arrows show the origin and direction of calcium waves initiated by the different pacemakers. (Left) PM1-confocal ratio imaging of cytosolic calcium (CG/TR) (1 image every 9 seconds). The ratios are shown as pseudocoloured images: higher $[Ca^{2+}]_c$ concentrations are in red and yellow; lower $[Ca^{2+}]_c$ in blue. The CG/TR ratio image sequence shows the initiation and propagation of the second calcium wave triggered by the moving series I pacemaker (PM1, white arrowhead at 4 minutes 0 seconds (4'00'')). All calcium waves in the series start in a peripheral ER-rich domain corresponding to the sperm aster (sa; arrowhead in TR image), which moves with the cortical contraction. The TR image shown (6'08'') corresponds to the third calcium wave initiated by pacemaker PM1. (Right) PM2-ratio CG/TR sequence of a series II calcium oscillation (1 image every 14 seconds). All waves are initiated from the same vegetal cortical region of the egg (contraction pole area; white arrowhead at t=12'15''). The sperm aster (sa, arrowhead in TR image) has enlarged and has moved into the interior of the egg away from the calcium wave pacemaker (PM2) located in the contraction pole which, in this case, is below the imaged confocal plane.



polar body (5-6 minutes after fertilization), the sperm aster and the growing spherical zone of ER accumulation around it move away from the zygote cortex (Fig. 1C; Fig. 2B, TR, PM2).

These observations demonstrate that, 2-3 minutes after sperm-egg fusion, the introduced sperm centrosome organizes a novel cortical and subcortical ER-rich domain.

Two physiological calcium wave pacemakers (PM1 and PM2) are associated with two distinct cortical ER-rich domains

The first pacemaker initiates series I calcium waves (Fig. 2). PM1 activity begins with a large fertilization or 'activation wave', which spreads through the whole egg from the point of sperm entry to the antipode (Speksnijder et al., 1990a; Roegiers et al., 1995). This activation wave is immediately followed by a series of 1-4 calcium waves (Fig. 2A) whose initiation sites progressively relocate towards the vegetal pole of the egg (McDougall and Sardet, 1995). We show here that the initiation sites of these waves lie in the most cortical region of the ER-rich domain associated with the sperm aster (Fig. 2B, PM1). This domain, which extends from the cortex to the subcortex of the egg (Fig. 1B; Fig. 2B, PM1) coincides with the location of sperm DNA (labelled with injected Hoechst 33258; data not shown).

Therefore the moving pacemaker that elicits series I calcium waves (PM1) is located first at the sperm entry site and then on the cortical side of the ER-rich sperm aster region.

After extrusion of the first polar body, calcium waves cease for 1-5 minutes depending on the species of ascidian (Speksnijder et al., 1989; Yoshida et al., 1998; Levasseur and McDougall, 2000). Calcium waves then start again from a different and stable location. During a period of 15-18 minutes, 5-12 waves that rise and then diminish in amplitude (Fig. 2A, PM2) emanate from a calcium wave pacemaker situated in the vegetal contraction pole (Speksnijder, 1992). This pacemaker (series II pacemaker: PM2) is located in a 2-6 μm -thick accumulation of cortical ER (McDougall and Sardet, 1995; Roegiers et al., 1999). As illustrated in Fig. 2B PM2, series II calcium waves clearly do not originate from the sperm aster which, by that time, has moved away from the egg cortex (observed in 20 different eggs). Also at this stage, the location of sperm DNA shows no preferred relationship with the vegetal contraction pole where calcium waves originate (data not shown).

Therefore, in ascidian zygotes, the two physiological pacemakers PM1 and PM2, which are responsible for eliciting series I and II calcium waves are both associated with cortical accumulations of ER.

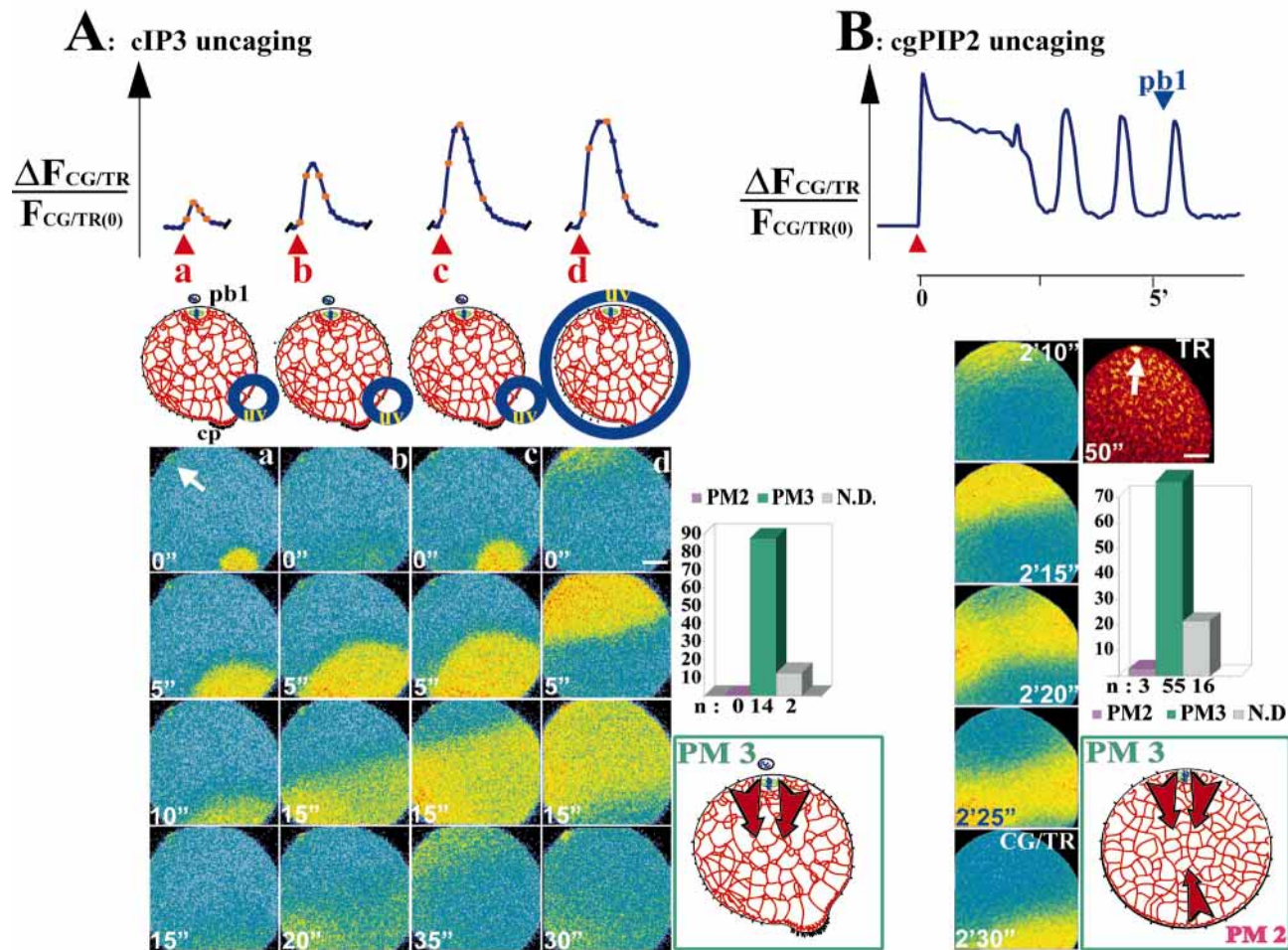


Fig. 3. An artificial calcium wave pacemaker (PM3) is generated by uncaging of cIns(1,4,5) P_3 or cgPtdIns(4,5) P_2 . (A) Variations of $[Ca^{2+}]_i$ induced by local and global UV photorelease of cIns(1,4,5) P_3 in activated eggs (1 image every 5 seconds; the images displayed in the CG/TR sequence correspond to the orange dots on the graph). The contraction pole (cp), first polar body (pb1, also indicated by a white arrow in a) and the size of the UV-flashed area (blue circle) are indicated. (a-c) Localized UV-flashes of increasing duration (1 second for a, 2 seconds for b, and 2.5 seconds for c) give rise to calcium waves initiated in the flashed area. The waves propagate further as increasing amounts of Ins(1,4,5) P_3 are photoreleased. (d) Brief (0.5 seconds) global UV-flashes over the whole egg increase the intracellular Ins(1,4,5) P_3 levels and give rise to calcium waves initiated in the egg cortex (1 image every 5 seconds). Bar, 23 μ m. The bar graph shows the number of waves that emanate from the animal hemisphere cortex (PM3) or the vegetal hemisphere cortex (PM2) after flash photolysis of cIns(1,4,5) P_3 . (B) Global UV photorelease of gPtdIns(4,5) P_2 in unfertilized eggs. A single UV-flash of long duration (red arrowhead, $t=0$) gives rise to calcium oscillations that resemble the physiological series I calcium oscillations and leads to the extrusion of pb1 (1 image every 10 seconds). CG/TR sequence: most gPtdIns(4,5) P_2 -induced calcium oscillations are waves initiated in the cortex of the animal pole region (see bar graph). The arrow in the TR image shows the meiotic spindle-associated ER-rich domain that marks the animal pole. Bar, 23 μ m.

A third pacemaker (PM3) can be induced by artificially raising Ins(1,4,5) P_3 levels in unfertilized eggs

To analyse the role of Ins(1,4,5) P_3 in regulating the repetitive calcium waves described above, we photoreleased cIns(1,4,5) P_3 and cgPtdIns(4,5) P_2 (a poorly metabolizable Ins(1,4,5) P_3 analogue (Bird et al., 1992)). Local or global increases in $[Ins(1,4,5)P_3]_i$ can be generated by either applying a brief UV-flash to a small area of the egg (Fig. 3Aa,b,c) or by exposing the whole egg to the UV-flash (Fig. 3Ad,B). Owing to frequent contamination of cIns(1,4,5) P_3 provided by manufacturers with uncaged Ins(1,4,5) P_3 , injection of cIns(1,4,5) P_3 often leads to an initial rise in $[Ca^{2+}]_i$, the formation of a contraction pole and the extrusion of the first polar body several minutes after injection (Fig. 3A). However, the injection does not trigger any

further calcium signals. Such artificially activated eggs can still respond in a graded manner to local release of increasing amounts of Ins(1,4,5) P_3 produced by localized UV-flashes (Fig. 3Aa,b,c). This graded response takes the form of calcium waves that spread further and further from the site of the UV-flash. Shorter flashes give rise to waves that do not cross the egg ('aborted' waves) (Fig. 3Aa,b), whereas longer flashes trigger waves that cross the whole egg (Fig. 3Ac).

A clear relationship between the amplitude and the speed of the calcium waves is observed during global uncaging of cIns(1,4,5) P_3 or cgPtdIns(4,5) P_2 over the whole egg, waves of highest amplitude being the fastest (data not shown). Initiation sites of calcium waves could only be resolved for slow waves resulting from brief pulses of UV light (Fig. 3Ad). Our analysis shows that 14 out of the 16 waves induced by global uncaging

of $c\text{Ins}(1,4,5)P_3$, emanated from the cortex in a broad area surrounding the animal pole of the egg (Fig. 3Ad, bar graph).

We could never generate more than a single calcium transient by uncaging $c\text{Ins}(1,4,5)P_3$. We therefore used $cg\text{PtdIns}(4,5)P_2$ to achieve a longer-lasting activation of the $\text{Ins}(1,4,5)P_3$ receptors ($\text{Ins}(1,4,5)P_3R$). $cg\text{PtdIns}(4,5)P_2$ injection does not activate eggs in most cases. Large increases in intracellular $g\text{PtdIns}(4,5)P_2$ trigger calcium oscillations that resemble series I calcium oscillations normally triggered by fertilization. They are characterized by a sustained increase in $[\text{Ca}^{2+}]_i$, followed by oscillations whose frequency decreases (compare Fig. 2A,3B). Such calcium signals are able to drive the first meiotic cell cycle, the first polar body being extruded on average 8 minutes and 10 seconds after the global UV-flash that uncages $cg\text{PtdIns}(4,5)P_2$ ($n=7$). The magnitude (amplitude and duration) of the initial calcium increase as well as the frequencies and duration of the calcium oscillations that follow vary with the amount of $g\text{PtdIns}(4,5)P_2$ photoreleased in the egg. Our analysis shows that 55 out of 58 oscillations induced by $g\text{PtdIns}(4,5)P_2$ are waves emanating from a broad peripheral area in the animal hemisphere (Fig. 3B, bar graph). The artificially induced calcium wave pacemaker PM3 remains in a stable location in the animal hemisphere (Fig. 3B). This is true even after a cortical contraction triggered by the elevation of $[\text{Ca}^{2+}]_i$ caused by $cg\text{PtdIns}(4,5)P_2$ photorelease. In this case a vegetal contraction pole similar to that produced after fertilization forms. Even when $g\text{PtdIns}(4,5)P_2$ is photoreleased in a small region in the vegetal hemisphere, which gives rise to a first wave starting in the flashed area, the calcium oscillations that follow are all waves triggered in the animal pole cortex (data not shown). These observations suggest that, after an initial localized UV-flash, the photoreleased $g\text{PtdIns}(4,5)P_2$ rapidly diffuses into the whole egg, homogenizing its intracellular concentration. This provides further evidence that the animal pole cortex is the area most sensitive to $\text{Ins}(1,4,5)P_3$. Finally, calcium influx seems not to play a role in the location of PM3, as similar results are obtained when $cg\text{PtdIns}(4,5)P_2$ uncaging experiments are performed in either normal sea water or calcium-free sea water that contains 2mM EGTA (data not shown).

Taken together, these results show that a stable artificial calcium wave pacemaker (PM3) can be induced in the animal pole cortex when $\text{Ins}(1,4,5)P_3$ or $g\text{PtdIns}(4,5)P_2$ levels are homogeneously elevated in unfertilized eggs.

The artificial pacemaker PM3 predominates over the physiological pacemaker PM2

Global increases of $g\text{PtdIns}(4,5)P_2$ levels can be generated in zygotes undergoing series II calcium waves (Fig. 4). When $cg\text{PtdIns}(4,5)P_2$ is uncaged homogeneously throughout the zygote, an artificial pacemaker PM3 is induced and initiates calcium waves (Fig. 4A). The physiological pacemaker PM2 can subsequently trigger calcium waves before the completion of meiosis II, as the effect of exogenous $g\text{PtdIns}(4,5)P_2$ is probably overcome by endogenously generated $\text{Ins}(1,4,5)P_3$ (Fig. 4A). Occasionally in a single zygote (Fig. 4B), three different types of calcium wave are induced by global uncaging of $cg\text{PtdIns}(4,5)P_2$ after the passage of a series II calcium wave (Fig. 4Bi,ii). A first UV-flash (uv1) elicits a calcium wave initiated by the artificial pacemaker PM3 in the animal cortex that spreads towards the vegetal hemisphere (Fig. 4B, uv1). After the passage of the UV-induced wave, the natural pacemaker PM2

then triggers a wave from the contraction pole (Fig. 4Bii). Although the vast majority of the artificially induced calcium waves come from the animal pole (83%, Fig. 4B, bar graph), a small proportion of the artificially induced waves (4 out of 53, Fig. 4, bar graph) originate from the vegetal contraction pole where the natural pacemaker PM2 is located (Fig. 4B, uv2). In a few cases (3 out of 53), artificial waves initiate almost simultaneously at both poles (Fig. 4B, uv3).

These results indicate that raising $\text{Ins}(1,4,5)P_3$ levels homogeneously throughout the zygote preferentially induces a stable artificial pacemaker located in the animal cortex (PM3). It is somewhat surprising that during the 20 minute period when the physiological pacemaker PM2 is operating in the vegetal contraction pole, the cortex of the animal hemisphere appears to remain the region of the zygote that is most sensitive to $\text{Ins}(1,4,5)P_3$.

The three pacemakers are differentially sensitive to cytoskeletal inhibitors

In order to further characterize and distinguish between the three calcium wave pacemakers, we tested their sensitivity to the microfilament and microtubule depolymerizing agents cytochalasin B and nocodazole. Nocodazole perfusion has been shown to depolymerize microtubules and prevent aster formation in *Phallusia mammillata* eggs but it does not affect the cortical contraction and the formation of a contraction pole rich in cortical ER (Roegiers et al., 1999; F. Roegiers, University of Nice, Nice, France, PhD thesis, 1999). We observed that perfusion of nocodazole during the activation wave prevented the formation of the subcortical ER-rich domain around the introduced sperm centrosome and its relocation away from the cortex (data not shown). Neither the location nor the activity of the three pacemakers seem to be affected by nocodazole treatment ($n=10$ for PM1 and PM2; $n=9$ for PM3; Fig. 5A,B).

Perfusion of 1 $\mu\text{g}/\text{ml}$ cytochalasin B on unfertilized eggs blocks the sperm-triggered cortical contractions, drastically altering sperm-triggered calcium waves (Fig. 5C). However, cytochalasin B treatment also induces polyspermy (in 7 out of 15 cases; data not shown). To avoid any interference with sperm-egg fusion, we perfused cytochalasin B during the propagation of the activation wave (Fig. 5D). The egg starts to contract but fails to complete the contraction. Within a minute of cytochalasin B perfusion, the egg rounds up again, indicating that the cortical microfilament organization had been disrupted. Under these conditions, the activity of PM1 and its movement are strongly altered, whereas the activation of PM2 is completely inhibited ($n=4$, Fig. 5D). If, however, cytochalasin B is perfused after the cortical contraction and extrusion of the first polar body, pacemaker PM2 is established and becomes insensitive to cytochalasin B ($n=2$, Fig. 5E). In contrast to the natural pacemakers PM1 and PM2, the artificial pacemaker PM3 could not be perturbed by cytochalasin B perfusion ($n=4$; Fig. 5B,F).

DISCUSSION

This study demonstrates for the first time that distinct ER-rich peripheral regions of the same egg cell can host different calcium wave pacemakers, generating stereotyped patterns of repetitive calcium waves. We also show that the animal pole

Fig. 4. The artificial pacemaker (PM3) predominates over the physiological calcium wave pacemaker (PM2). (A) Variations of $[Ca^{2+}]_c$ in a fertilized egg undergoing meiosis II (1 image every 10 seconds), in response to a global increase in $gPtdIns(4,5)P_2$ levels (red arrows indicate the four successive 1.5-second UV flashes applied). The flashes produce a sustained calcium increase, then two oscillations that are waves initiated in the animal pole (PM3, green graph). The three subsequent waves emanate from pacemaker PM2 in the contraction pole (pink graph) and precede the extrusion of the second polar body (pb2). (B) Another experiment showing the effect of $cgPtdIns(4,5)P_2$ uncaging during the series II calcium waves (1 image every 4 seconds). The images displayed in the CG/TR sequence correspond to the yellow dots on the graph. The physiological series II calcium wave pacemaker PM2 is located in the contraction pole (cp) after PM1 activity has ceased (calcium waves shown in i and ii; Bar, 23 μm). This particular experiment shows that global UV-flashes over the whole zygote (uv1, uv2, uv3) can elicit three different types of waves. The first global photorelease of $gPtdIns(4,5)P_2$ (uv1, flash duration 3 seconds; red arrowhead at 7'19'') triggers an artificial calcium wave whose initiation site (PM3) is in the animal pole (a) cortex. The second UV-flash (uv2, flash duration 1 second; red arrowhead at 11'17'') triggers an artificial calcium wave initiated in the contraction pole in the vegetal hemisphere. The third UV-flash (uv3, flash duration 2 seconds, arrowhead at 13'49'') elicits a calcium wave that is initiated in both the animal and vegetal pole regions. Probabilities for the occurrence of each type of wave are shown in the bar graph.

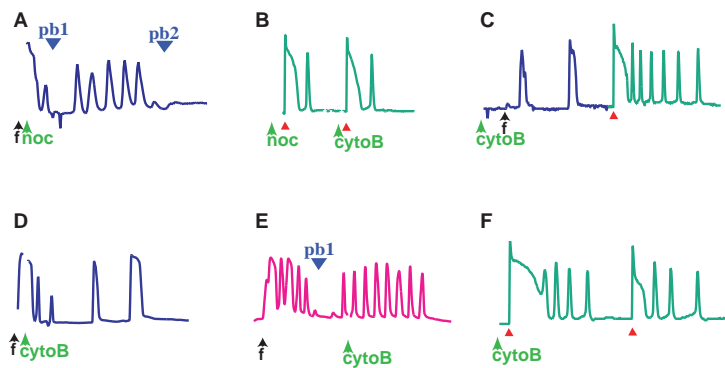
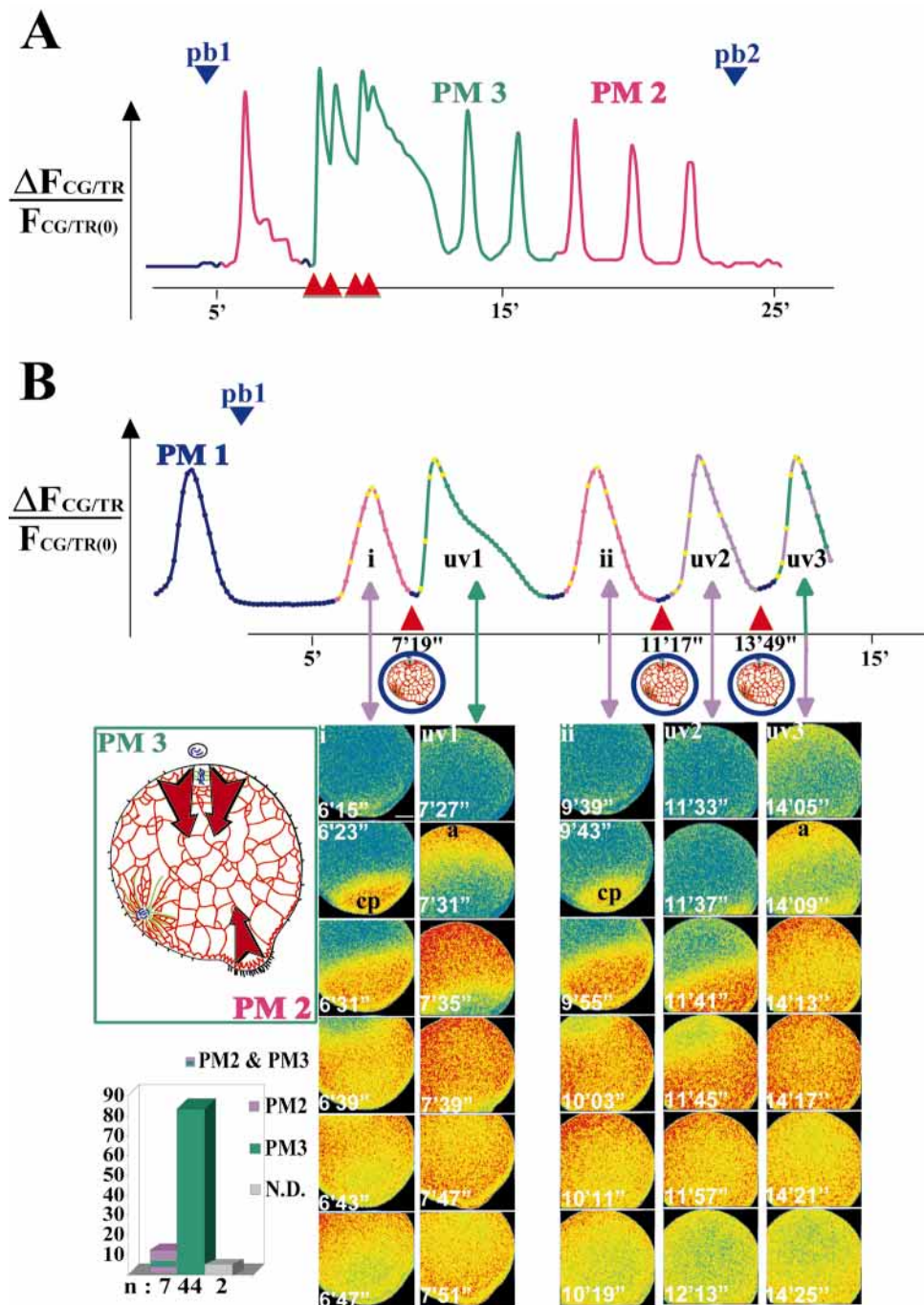


Fig. 5. Differential sensitivity of the three pacemakers to cytoskeletal inhibitors. Patterns of $[Ca^{2+}]_c$ oscillations triggered by fertilization (A,C-E) or after photorelease of $cgPtdIns(4,5)P_2$ (B,C,F) in ascidians eggs perfused with nocodazole (A,B) or cytochalasin B (C-F) or both (B). The green arrowheads indicate the time of perfusion of the cytoskeletal inhibitors nocodazole (noc) or cytochalasin B (cytoB). Interruptions in the graphs are due to the fact that no measurements could be made during the time of perfusion. The black arrowheads mark the time of fertilization (f), while the red arrowheads indicate the time at which a UV flash is applied to uncage $cgPtdIns(4,5)P_2$.

cortex of the egg is the area most sensitive to $\text{Ins}(1,4,5)P_3$ and it can act as an artificial calcium wave pacemaker during sustained increases in $\text{Ins}(1,4,5)P_3$ levels. Surprisingly, this artificial pacemaker predominates over the natural pacemaker PM2 situated in the vegetal cortex.

The physiological pacemakers PM1 and PM2 are located in ER-rich cortical domains and require factor(s) brought by sperm

The abundant ER network in the ascidian zygote is the main $\text{Ins}(1,4,5)P_3$ -sensitive calcium store. It is organized into ER-rich domains or clusters that grow in size after fertilization (Spesknijder et al., 1993; Roegiers et al., 1999). The ER-rich domains may create amplification sites that favour calcium wave initiation and propagation because of the better coordination of elementary calcium signals (puffs) in these cytoplasmic domains (Berridge, 1997; Stricker et al., 1998; Kline et al., 1999; Kline, 2000; Marchant and Parker, 2001).

Pacemaker PM1 forms at the site of sperm-egg fusion and subsequently moves towards the vegetal hemisphere. We clearly show here that PM1 activity moves with the cortical and subcortical ER-rich domain that gathers in the sperm aster region (Fig. 2B). The motion of PM1 towards the vegetal hemisphere is undoubtedly due to the acto-myosin-driven cortical contraction triggered by the activating calcium wave. The contraction also relocates and concentrates the cortical ER in the contraction pole (Roegiers et al., 1995; Roegiers et al., 1999). The cortical contraction and the displacement of PM1 are blocked by the microfilament depolymerizing agent cytochalasin B (Speksnijder et al., 1990a; this study). This treatment dramatically alters PM1 activity (Fig. 5), which suggests that either the motion of the sperm aster or an intact cortical microfilament network are critical for PM1 activity. Nocodazole treatment further demonstrates that the subcortical ER-rich domain that forms around the sperm centrosome is not required per se for the activity of the moving pacemaker PM1.

Pacemaker PM2 takes over from pacemaker PM1 near the time of meiosis I completion. At the time of polar body extrusion, when MPF is low, PM2 activity is suppressed (Levasseur and McDougall, 2000). As MPF rises again, calcium waves of larger and larger amplitude are then triggered by PM2 from the vegetal contraction pole: a constriction 15–20 μm in diameter that forms in the vegetal hemisphere as a result of the acto-myosin driven cortical contraction. Within the contraction pole, PM2 activity is situated in a 2–6 μm -thick cortical zone of ER accumulation sandwiched between a tuft of microvilli and a 7–10 μm -thick mitochondria-rich domain (McDougall and Sardet, 1995; Roegiers et al., 1999).

Treatment with cytochalasin B at the beginning of cortical contraction inhibits the activation of PM2 (Fig. 5). In this case, the contraction pole containing the accumulation of cortical ER does not form. However if cytochalasin is perfused just after the extrusion of the first polar body, an ER-rich cortical layer has had time to form in the vegetal hemisphere and, although the egg rounds up, pacemaker PM2 is activated and emits a normal series of waves until the completion of meiosis II. This suggests that an ER accumulation in the contraction pole is necessary for PM2 to function.

However, this accumulation of cortical ER in the contraction

pole is not sufficient in itself to initiate and sustain PM2 activity. Indeed, even though global elevations in calcium or $\text{Ins}(1,4,5)P_3$ result in the formation of a contraction pole that contains the characteristic ER accumulation, microvilli and microfilaments, this ER-rich cortical domain is not the site of artificial pacemaker PM3. This indicates that the activity of the physiological pacemaker PM2 must rely on additional factors besides cortical ER accumulation and global elevation in the level of $\text{Ins}(1,4,5)P_3$.

These additional factors are probably brought by the sperm, as injection of sperm extracts in ascidian eggs can effectively induce the two physiological pacemakers PM1 and PM2 (Kyojuka et al., 1998; McDougall et al., 2000; Runft and Jaffe, 2000). The nature of the factor brought by sperm is still controversial. Current candidates include a protein activator of phospholipase C (PLC) in ascidians (Runft and Jaffe, 2000), an activated PLC in mammals (Jones et al., 2000; reviewed by Swann and Parrington, 1999) and other possible proteins in echinoderms (Kuo et al., 2000).

The animal pole cortex is most sensitive to $\text{Ins}(1,4,5)P_3$ and can act as an artificial pacemaker (PM3)

Global flash photolysis of $\text{cIns}(1,4,5)P_3$ and $\text{cgPtdIns}(4,5)P_2$, produces waves emanating from pacemaker PM3 located in a broad peripheral area in the animal hemisphere. Because it does not depend on the presence of external calcium, this higher sensitivity of the animal hemisphere to a global rise in $\text{Ins}(1,4,5)P_3$ probably relies on the presence of numerous ER-rich domains in the animal cortex and subcortex of the egg (Fig. 1). Similarly, the cortex of unfertilized mouse eggs shows a higher sensitivity to $\text{Ins}(1,4,5)P_3$ compared with the interior of the egg (Oda et al., 1999).

As discussed above, global calcium waves result from the activation and co-ordination of elementary events (Ca^{2+} 'puffs') (Berridge, 1997; Marchant and Parker, 2001). Interestingly, in *Xenopus* oocytes, when $\text{Ins}(1,4,5)P_3$ is photoreleased, the calcium puffs elicited in the animal hemisphere are bigger and closer to each other than in the vegetal hemisphere. However, this asymmetry does not seem to result in the preferential initiation of global calcium waves in the animal hemisphere (Callamaras et al., 1998). In *Xenopus* oocytes a sustained elevation in $\text{Ins}(1,4,5)P_3$ levels induces repetitive calcium waves that originate from a small number of specific 'focal puff' sites particularly sensitive to $\text{Ins}(1,4,5)P_3$ (Marchant and Parker, 2001). We do not know if such microscopic focal puff sites exist in ascidian eggs. On the macroscopic level, the presence of a mitochondria-rich and ER-poor subcortical region in the vegetal hemisphere may be critical for creating the functional heterogeneity necessary to induce PM3 activity and the preferential initiation and propagation of global calcium waves in the animal hemisphere of the egg.

We have repeatedly observed that the artificial pacemaker PM3 in the animal pole predominates over the physiological PM2 when $\text{Ins}(1,4,5)P_3$ (or $\text{gPtdIns}(4,5)P_2$) levels are homogeneous throughout the egg (Fig. 4). This suggests that, in contrast to somatic cells, the particular spatiotemporal characteristics of the rise in cytosolic $\text{Ins}(1,4,5)P_3$ levels in ascidian zygotes are likely to be important for the definition of the pacemaker sites (see below).

Different spatiotemporal patterns of [Ins(1,4,5) P_3]c may generate distinct cortical calcium wave pacemakers in ascidian zygotes

Ins(1,4,5) P_3 is thought to be the main secondary messenger involved in egg activation and in the regulation of the meiotic calcium oscillations in most species (reviewed by Sardet et al., 1998; Stricker, 1999; Swann and Parrington, 1999; Kline, 2000). In ascidians, although there is evidence for the presence of ryanodine receptors (RyR) in the cortex of the egg, they apparently do not play any role in the regulation of the sperm-triggered calcium oscillations (Albrieux et al., 1997; Yoshida et al., 1998). Similarly, NAADP, which desensitizes Ins(1,4,5) P_3 -mediated calcium signalling in ascidians, is thought not to participate in the regulation of the sperm-triggered calcium oscillations (Albrieux et al., 1998).

The artificial pacemaker PM3 produced by uncaging cgPtdIns(4,5) P_2 globally in the activated egg (Fig. 3) or zygote (Fig. 4) elicits calcium oscillations with similar temporal characteristics to those generated by PM1. This strongly suggests that a single, large and sustained increase in Ins(1,4,5) P_3 levels regulates the physiological pacemaker PM1.

If their temporal characteristics are similar, the spatial characteristics of PM3 (broad and stable pacemaker) and PM1 (focused and moving pacemaker) are, however, very different. Considering that any rise in Ins(1,4,5) P_3 throughout the egg above a critical level triggers waves from the animal pole pacemaker PM3, the most likely explanation for the moving pacemaker PM1 is that it is due to a localized production of Ins(1,4,5) P_3 at the pacemaker site. Our current hypothesis is that a highly active sperm factor located in the site of sperm-egg fusion and then in the moving sperm aster region generates the high rate Ins(1,4,5) P_3 that sustains PM1 activity.

PM1 stops operating just before the expulsion of the first polar body, when low MPF allows for the growth of the microtubules, which can push the ER-rich sperm centrosome away from the cortex. The delocalization of the sperm aster ER-rich domain does not seem to play a major role in the switching between PM1 and PM2 activity, since there are no changes in the pattern of calcium waves when this delocalization is prevented by nocodazole treatment.

In ascidians, early and late calcium waves that emanate from the physiological pacemaker PM2 are of lower amplitude and are typically aborted (McDougall and Sardet, 1995). We could generate similar aborted waves in the vegetal cortex by local photorelease of Ins(1,4,5) P_3 in the vegetal hemisphere (Fig. 3) but not by global Ins(1,4,5) P_3 increases which, in the vast majority of cases, induce PM3. This leads us to suggest that PM2 activity in the vegetal contraction pole also relies on a localized production of Ins(1,4,5) P_3 . The vegetal contraction pole is a special region of the cortex characterized by a tuft of microvilli (Roegiers et al., 1995; Roegiers et al., 1999). We postulate that this plasma membrane-enriched region represents a concentrated source of membranous PtdIns(4,5) P_2 and potential source of Ins(1,4,5) P_3 in close proximity to the 2–6 μm -thick layer of cortical ER. This dense accumulation of ER adhering to the plasma membrane would represent a tightly coupled target of locally generated Ins(1,4,5) P_3 , and the site of the initial calcium release events (focal puffs) that trigger each calcium wave in the contraction pole.

It is not clear at present whether a slow continuous

production of Ins(1,4,5) P_3 or repetitive elevations in Ins(1,4,5) P_3 levels in the contraction pole underly PM2 activity. In mouse eggs it has been shown that long-lasting calcium oscillations displaying similar temporal characteristics to those produced by sperm, can be generated through the continuous photorelease of cIns(1,4,5) P_3 (Jones and Nixon, 2000). Similarly, the ascidian PM2 activity may rely on a continuous low production of Ins(1,4,5) P_3 in the contraction pole somehow leading to a stable [Ins(1,4,5) P_3]c gradient necessary for the repetitive firing of calcium waves from that site. Alternatively, a pulsatile and localized generation of Ins(1,4,5) P_3 could create the gradient of Ins(1,4,5) P_3 concentration necessary to maintain PM2 activity at a much smaller energy cost. It is known that the prolonged stimulation of a calcium-activated PLC can result in [Ins(1,4,5) P_3]c oscillations (see Meyer and Stryer, 1988 for details). Considering that, the sperm factor is thought to be a PLC activator in ascidians (Runft and Jaffe, 2000) and a calcium-activated PLC in mammals (Swann and Parrington, 1999; Rice et al., 2000), the prolonged stimulation of PLC during meiosis II in ascidians could indeed result in a pulsatile production of Ins(1,4,5) P_3 . Measurements of Ins(1,4,5) P_3 levels in single living eggs are now needed to find out whether Ins(1,4,5) P_3 production is continuous or pulsatile.

Polarized calcium signals and the animal-vegetal polarity of eggs and zygotes

Stable calcium wave pacemakers residing within cytoplasmic areas rather than in the immediate vicinity of the plasma membrane have been characterized in some somatic cells (Ito et al., 1999; Thomas et al., 1999). In pancreatic acinar cells the 'trigger zone' is an ER-rich zone in the apical region where type 3 Ins(1,4,5) P_3 R are abundant (reviewed by Petersen et al., 1999). In HeLa cells, 'pacemaker Ca^{2+} puffs sites' with stable perinuclear locations are at the origin of the initiation of a global calcium wave upon stimulation. In these cells, the physiological pacemaker site apparently coincides with the area of the cell that is most sensitive to Ins(1,4,5) P_3 . In neurite-bearing cells, the calcium wave pacemaker site is regulated by the shape of the cell itself and there is no need for a local source of Ins(1,4,5) P_3 production to induce Ins(1,4,5) P_3 waves and the stable calcium wave pacemaker (Fink et al., 1999). In contrast to somatic cells, we believe that, in ascidian eggs and zygotes, both cortical ER accumulations and local production of Ins(1,4,5) P_3 are required for the generation of repetitive calcium waves by the physiological pacemakers.

It has been known for a decade that in the ascidian zygote, repetitive calcium waves are generated from a cortical ER accumulation in the vegetal contraction pole (Speksnijder et al., 1990b). Similarly, in the mouse, it has been discovered only recently that meiosis II-associated oscillations originate from the vegetal cortex where ER clusters are situated (Kline et al., 1999; Deguchi et al., 2000). A vegetal calcium wave pacemaker that generates repetitive calcium waves associated with meiosis I has also been observed in the nemertean *Cerebratulus lactus* (Stricker, 1996; Stricker et al., 1998). The fact that, in nemerteans, mouse and several species of ascidians, repetitive waves originate in the vegetal cortex, suggests that vegetal calcium wave pacemakers could be an evolutionarily conserved feature in protostome and deuterostome early development. We may wonder whether this

is a mechanism used to prime the vegetal pole region for later developmental events. In nemerteans and ascidians the vegetal pole region corresponds to the future site of gastrulation of the embryo.

It has been suggested that the frequency and amplitude of repetitive calcium waves affect development in mammals (Jones, 1998; Lawrence et al., 1998; Ozil, 1998). The possibility of generating an artificial pacemaker (PM3) at the antipode of the physiological pacemaker (PM2) provides a unique opportunity to investigate the importance of the spatial pattern of calcium waves in the development of the relatively simple ascidian tadpole.

In conclusion, our work on ascidian zygotes supports the idea that complex spatiotemporal patterns of $\text{Ins}(1,4,5)\text{P}_3$ can induce different calcium wave pacemakers in the same cell. We suggest that the physiological pacemakers driving meiosis I and II may necessitate the close apposition of ER-rich domains (the target of $\text{Ins}(1,4,5)\text{P}_3$) to localized sources of $\text{Ins}(1,4,5)\text{P}_3$ production, which are likely to result from the activity of sperm factors. Future studies should reveal whether similar mechanisms are at work in zygotes of other species.

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