

Review

Fertilisation calcium signals in the ascidian egg

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Abstract

The egg of ascidians (urochordate), as virtually all animal and plant species, displays Ca^{2+} signals upon fertilisation. These Ca^{2+} signals are repetitive Ca^{2+} waves that initiate in the cortex of the egg and spread through the whole egg interior. Two series of Ca^{2+} waves triggered from two distinct Ca^{2+} wave pacemakers entrain the two meiotic divisions preceding entry into the first interphase. The second messenger inositol (1,4,5) trisphosphate (IP3) is the main mediator of these global Ca^{2+} waves. Other Ca^{2+} signalling pathways (RyR and NAADPR) are functional in the egg but they mediate localised cortical Ca^{2+} signals whose physiological significance remains unclear. The meiosis I Ca^{2+} wave pacemaker is mobile and relies on intracellular Ca^{2+} release from the endoplasmic reticulum (ER) induced by a large production of IP3 at the sperm aster site. The meiosis II Ca^{2+} wave pacemaker is stably localised in a vegetal protrusion called the contraction pole. It is probable that a local production of IP3 in the contraction pole determines the site of this second pacemaker while functional interactions between ER and mitochondria regulate its activity. Finally, a third ectopic pacemaker can be induced by a global increase in IP3, making the ascidian egg a unique system where three different Ca^{2+} wave pacemakers coexist in the same cell.

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1. Introduction

Ascidians (tunicates/urochordates) are sessile marine organisms whose egg develops into tadpole larvae that have the basic body plan of chordates. In most species, sperm entry is accompanied by an increase in the Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) that invades the whole egg and activates the development of the egg. In ascidians, as in nemerteans, some molluscs and mammals, the fertilisation Ca^{2+} wave is followed by a series of repetitive Ca^{2+} waves driving the destruction of maturation promoting factor (MPF) and completion of meiosis before the pronuclei can form (McDougall and Levasseur, 1998; Stricker, 1999; Nixon et al., 2000; Carroll, 2001; Dumollard et al., 2002). Eggs of mammals are arrested in metaphase II and a minimum number of Ca^{2+} transients is necessary for establishment of a block to polyspermy, recruitment of maternal RNAs and entry into first interphase (Ducibella et al., 2002). Eggs of ascidians that are arrested at metaphase I require two series of Ca^{2+} waves to complete two meiotic divisions (McDougall and Sardet,

1995; Yoshida et al., 1998). These meiotic Ca^{2+} waves also stimulate mitochondrial ATP production to match the increased energy demand associated with the onset of development (Dumollard et al., 2003).

During maturation, which consists of cytoplasmic growth and establishment of a polarised cortex and subcortex, the egg acquires the ability to respond to the fertilising sperm by eliciting repetitive Ca^{2+} waves. The ascidian egg possesses a complex Ca^{2+} signalling machinery hosted by the plasma membrane, the endoplasmic reticulum (ER) and mitochondria. The interior of the mature, metaphase I-arrested egg is organised into cortical and cytoplasmic domains made of different concentrations of intracellular organelles (the three main ones being ER, mitochondria and yolk platelets, Speksnijder et al., 1993; Roegiers et al., 1999; Dumollard and Sardet 2001). The Ca^{2+} waves are initiated in the cortex and they spread through the subcortex and the deeper cytoplasm of the egg. Because each of these subcellular regions hosts a different proportion of Ca^{2+} -releasing organelles the egg interior is an inhomogeneous medium for the propagation of a Ca^{2+} wave.

The fertilisation Ca^{2+} wave triggers an actomyosin-driven contraction that reorganises the cortex and subcortex of the

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egg to form a vegetal constriction called the contraction pole, a specialised domain dedicated to the initiation of meiotic Ca^{2+} waves (Speksnijder et al., 1993; McDougall and Sardet, 1995; Roegiers et al., 1995; Roegiers et al., 1999; Dumollard et al., 2002). A first Ca^{2+} wave pacemaker functions during this cortical contraction and it is translocated towards the vegetal pole of the egg. After a lag time during which the first polar body is extruded, a second pacemaker appears in the newly formed contraction pole. The ascidian zygote was the first example of a non-excitable cell where two physiological Ca^{2+} wave pacemakers exist successively; this phenomenon has now also been observed during mammalian fertilisation (Deguchi et al., 2000). A third artificial pacemaker can be induced in ascidian eggs and it can compete with the natural pacemakers induced by fertilisation (Dumollard and Sardet, 2001; Dumollard et al., 2003). The occurrence in the same cell of two distinct pacemakers both regulated by IP_3 raises the question of how a highly diffusible second messenger like IP_3 can give rise to distinct Ca^{2+} wave pacemakers with distinct spatio-temporal characteristics in a cell.

The large size of the ascidian egg, its transparency and the occurrence of several physiological Ca^{2+} wave pacemakers at fertilisation make it a unique system to observe intracellular Ca^{2+} wave pacemakers, identify their constituents and decipher the mechanisms of initiation and propagation of intracellular Ca^{2+} waves. After reviewing the different Ca^{2+} release pathways characterised in ascidian egg, we will then describe the intracellular organisation of the Ca^{2+} releasing organelles. We also examine how the intrinsic organisation of the egg cytoplasm, coupled with factors introduced by the sperm, support the activity of these two Ca^{2+} wave pacemakers that are distinct in their origin and their mode of action. Finally the role of a vegetal Ca^{2+} wave pacemaker on the early development of the embryo will be discussed.

2. The Ca^{2+} signalling machinery of the egg

The two main sources of Ca^{2+} in the egg lie in the ER stores and Ca^{2+} influx through the plasma membrane, while mitochondria can sequester some of the released Ca^{2+} (Table 1). The first noticeable sign of fertilisation is the appearance of fertilisation currents that provoke depolarisation of the plasma membrane (Goudeau et Goudeau, 1993). This depolarisation activates voltage operated Ca^{2+} channels

(VOCC) on the egg plasma membrane (Arnoult et al., 1996; Albrieux et al., 1997). Recently, a stretch-activated TRPV Ca^{2+} channel has been cloned in *Halocynthia roretzi* (Kondoh et al., 2003); such a Ca^{2+} channel may be activated by the cortical contraction deforming the plasma membrane during the fertilisation Ca^{2+} wave. Ca^{2+} release activated Ca^{2+} (CRAC) channels have also been characterised in the ascidian egg (Arnoult et al., 1996; Albrieux et al., 1997). Therefore there are three different routes for external Ca^{2+} to flow into the cytosol but, as none of these pathways is able to increase $[\text{Ca}^{2+}]_c$ by itself, they may only be used to refill the Ca^{2+} stores. In addition, even though Ca^{2+} influx may occur during the operation of the pacemakers, it has been shown that the second phase of Ca^{2+} oscillations does not require external Ca^{2+} (Sensui and Morisawa 1996; Carroll et al., 2003) raising the question of the physiological significance of Ca^{2+} influx into the fertilised egg. A 40 % increase in plasma membrane area takes place at fertilisation and microvilli grow during the establishment of the contraction pole (Albrieux et al., 1998; Albrieux et al., 2000; Sardet et al., 2002). There is no cortical granule exocytosis in ascidian eggs and it is not known which intracellular membranous organelle fuses with the plasma membrane. Whether the new membrane brings new channels to the plasma membrane or whether such membrane insertion dilutes the existing population of channels remains to be established.

Three pathways of intracellular Ca^{2+} release have been characterised in the ascidian egg: they are the IP_3 receptor, the ryanodine receptor (RyR) and the nicotinic acid-adenine dinucleotide phosphate (NAADP) Ca^{2+} releasing system. (McDougall et Sardet, 1995; Albrieux et al., 1998; Albrieux et al., 2000; Dumollard et al., 2002; Table 1). In the ascidian genome only one IP_3 receptor type corresponding to type I can be found but the three types of ryanodine receptors are present.

Every part of the cytosol is able to respond to IP_3 and supports an IP_3 -mediated Ca^{2+} wave indicating that IP_3Rs are located on the whole ER network (Roegiers et al., 1995; Dumollard and Sardet, 2001; table 1). In contrast, immunostaining of *P. mammillata* eggs revealed that the RyR is present only in the vegetal cortex of the egg (Albrieux et al., 2000) while NAADPR is located on a cortical organelle yet unidentified (Albrieux et al., 2000). So far, IP_3 but not cAD-Prribose (gating RyR) nor NAADP is able to induce a global Ca^{2+} wave demonstrating that the IP_3R is the most important

Table 1

Type and localisation of the Ca^{2+} signalling machinery in the ascidian egg.

The pathways for Ca^{2+} influx into the cytosol and Ca^{2+} efflux from the cytosol are indicated as well as their organellar origin and their presumed localisation in the egg. VOCC : Voltage-operated Ca^{2+} channel; CRAC : Ca^{2+} release activated Ca^{2+} ; PMCA : Plasma membrane Ca^{2+} ATPase; IP_3R : IP_3 receptor; RyR : Ryanodine receptor; NAADPR : NAADP receptor; SERCA : Sarco-endoplasmic reticulum Ca^{2+} ATPase; Ca^{2+} unip.: Ca^{2+} uniporter; $\text{Na}^+/\text{Ca}^{2+}$ exch.: $\text{Na}^+/\text{Ca}^{2+}$ exchanger; TRPV : transient receptor potential V.

organelle		plasma membrane	endoplasmic reticulum		unidentified organelle	mitochondria
Ca^{2+} signalling machinery	influx	VOCC CRAC channel TRPV channel	IP_3R	RyR	NAADPR	$\text{Na}^+/\text{Ca}^{2+}$ Exch.
	efflux	PMCA	SERCA	SERCA		Ca^{2+} Unip.
localisation		animal & vegetal cortex	whole egg	vegetal cortex	animal & vegetal cortex	mostly vegetal subcortex

intracellular Ca^{2+} release channel in the ascidian egg. The predominant role of IP₃ in mediating meiotic Ca^{2+} waves has been established in the ascidian egg and is reviewed elsewhere (Sardet et al., 1998; Nixon et al., 2000; Dumollard et al., 2002). NAADP and RyR pathways would be involved only in the generation of localised Ca^{2+} signals in the cortex of the egg. The RyR mediated Ca^{2+} signal is necessary for new membrane insertion whereas the physiological significance of NAADP induced Ca^{2+} release remains unknown (Albrieux et al., 1998; Albrieux et al., 2000). As every meiotic Ca^{2+} wave in the ascidian starts from the cortex where these receptors are presumably located, it is possible that RyR and NAADP participate somehow in the initiation of the global Ca^{2+} wave in the egg.

In somatic cells, mitochondria have been shown to induce both negative and positive feedback on IP₃-mediated Ca^{2+} signals (Duchen 2000; Rizzuto et al., 2000). There is a dynamic interplay between ER Ca^{2+} release sites and mitochondria that buffer cytosolic Ca^{2+} in the vicinity of the IP₃R thereby modulating the opening of the IP₃R. Mitochondria also provide ATP that can bind the IP₃R or be used to refill the ER stores. We found that in the ascidian egg mitochondria sequester Ca^{2+} during each wave and that mitochondrial respiration is stimulated by these Ca^{2+} waves (Dumollard et al., 2003). Switching off mitochondria using different mitochondrial inhibitors has a very minor effect on the first Ca^{2+} wave pacemaker (PM1) whereas it blocks rapidly and completely the second pacemaker (PM2). This difference in the sensitivity of the two pacemakers to mitochondrial inhibitors may be explained by the different amount of IP₃ that regulate PM1 and PM2. Indeed, a much larger amount of IP₃ regulates PM1 compared to PM2 which requires a low level of IP₃ (Dumollard and Sardet, 2001). Under the high IP₃ levels underlying PM1 activity, the action of mitochondria is accessory. In contrast, both sequestering of cytosolic Ca^{2+} by mitochondria and mitochondrial ATP production are necessary for PM2 to function (Dumollard et al., 2003). Ca^{2+} buffering by mitochondria may retard the closure of IP₃R by high Ca^{2+} ; at the same time the ATP produced can bind to nearby IP₃R and sensitise the IP₃R to Ca^{2+} or it can be used by Ca^{2+} pumps of the ER to refill the stores. Ca^{2+} buffering by mitochondria will lead to a larger release of Ca^{2+} into the cytosol and mitochondrial ATP production will lower the threshold of activation of the IP₃R by Ca^{2+} . Accordingly, uncaging ATP in the egg during the operation of PM2, does not modify the amplitude of the Ca^{2+} wave but it increases the frequency of the oscillations suggesting that the mitochondrial ATP production sensitises the IP₃R to activation by Ca^{2+} . As elevated ATP levels will affect a number of cellular processes it is also possible that the functioning of the sperm factor and therefore the production of IP₃ are affected by elevated ATP levels. This approach cannot distinguish between an action of ATP directly on the IP₃R and an action via stimulation of the ER Ca^{2+} pumps or of the sperm factor. Nevertheless, mitochondria seem to modulate the temporal pattern of the Ca^{2+} waves emitted by PM2.

The ascidian egg shows a very specific distribution of this complex system of Ca^{2+} releasing organelles. The cortical and cytoplasmic domains constituted by these organelles are established during maturation and reorganised after fertilisation. These domains represent a local environment with a different excitability with regards to Ca^{2+} release. During their progression through the egg cytoplasm the Ca^{2+} waves will nevertheless propagate efficiently through these distinct layers.

3. Organisation of the Ca^{2+} signalling machinery

The cortical and cytoplasmic domains display an asymmetric distribution along a radial axis as well as along the animal-vegetal (AV) axis of the egg. The organelles are naturally stratified and three main regions can be distinguished: they are the cortex, the subcortex and the deeper cytoplasm (Fig. 1).

The egg cortex is a specialisation of the egg cytoplasm dedicated to the initiation of global Ca^{2+} waves. It comprises the plasma membrane and underlying layers of microfilaments and cortical ER (Sardet et al., 2002). The contractile meshwork of microfilament is depleted in the animal pole region which is occupied by the meiotic spindle embedded in a large cortical ER-rich domain (Speksnijder et al., 1993; Dumollard and Sardet, 2001). After fertilisation, the monolayer of cortical ER present as an animal-vegetal gradient in the unfertilised egg (Sardet et al., 1992) accumulates in the vegetal hemisphere forming a 2–5 μm thick ER-rich domain behind the plasma membrane (Speksnijder et al., 1993; Rogiers et al., 1999; Dumollard and Sardet 2001, Sardet et al., 2003). The egg cortex is a region of higher sensitivity to IP₃

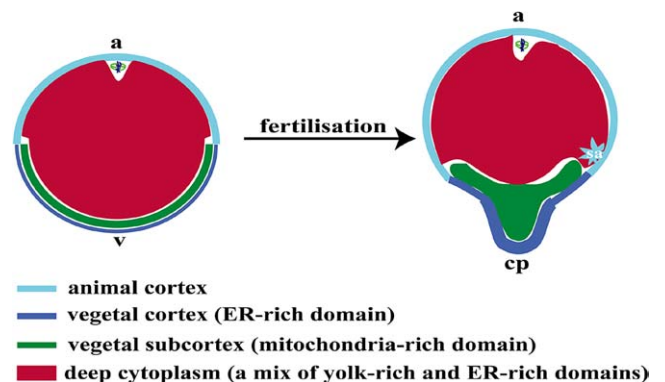


Fig. 1. Cytoplasmic domains in the ascidian egg.

A schematic representation of the egg shows the main cortical and cytoplasmic domains with: the animal cortex in light blue, vegetal cortex in dark blue, mitochondria-rich vegetal subcortex (called myoplasm) in green and deeper cytoplasm made of ER-rich and yolk-rich microdomains in red. The fertilisation Ca^{2+} wave triggers a wave of cortical contraction which reorganises the egg cortex and subcortex. A constriction called the contraction pole (cp) is formed in the vegetal hemisphere of the egg by this cortical contraction. The contraction pole has a thicker ER-rich cortex (dark blue) and mitochondria-rich subcortex (green) as cortical and subcortical domains have been dragged with the cortical contraction and have accumulated in and around the contraction pole. a: animal pole; v: vegetal pole; sa: sperm aster.

because of the presence of cortical ER-rich domains that are concentrated in the layer abutting the plasma membrane (represented in blue in Fig. 1). Since the plasma membrane contains the IP₃ precursor PIP₂ it is expected that the cortex is the major site of IP₃ production and the site of initiation of global Ca²⁺ waves. Consistent with this, all the natural global Ca²⁺ waves occurring in the egg initiate in different regions of the egg cortex.

After initiation in the cortex, the Ca²⁺ waves spread through the subcortex and the deeper cytoplasm. An extensive ER network made of branched microdomains of ER accumulation invades the whole egg except in the mitochondria-rich vegetal subcortex (Dumollard and Sardet, 2001). In this subcortical domain called the “myoplasm” (represented in green in Fig. 1) the mitochondria are so densely packed that only ER tubules and very sparse ER-rich domains traversing the myoplasm can be observed (Speksnijder et al., 1993; Dumollard and Sardet, 2001; Dumollard et al., 2003; fig. 3A). Imaging Ca²⁺ with a cytosolic dye reveals striking differences between the mitochondria-rich vegetal subcortex (Fig. 3A) and the deeper cytoplasm (Fig. 3B). A confocal image of the cytosol in the mitochondria-rich domain shows a punctate pattern that is due to the presence of densely packed mitochondria which exclude the cytosolic dye (Fig. 3A). Yolk platelets are seen as larger dark vesicles in the confocal image (Fig. 3). In this mitochondria-rich domain the Ca²⁺ wave propagates with a constant speed before slowing down (Fig. 3A). Transduction of the wave in this domain involves cycles of Ca²⁺ release from the ER and probably from the mitochondria themselves. Analysis of Ca²⁺ wave propagation in the mitochondria-rich domain in the presence of a specific blocker of mitochondrial Ca²⁺ uniporter should reveal whether they are passive or active participants in the transduction of the Ca²⁺ wave. In addition, the presence of ER-rich domains does not seem necessary per se for the propagation of the Ca²⁺ wave. Only the presence of ER tubules is required for the propagation of a Ca²⁺ wave in this mitochondria-rich domain raising the question of the physiological significance of the ER-rich domains.

The deeper cytoplasm shows a more reticular pattern with ER-rich domains (containing a higher concentration of cytosol, see Dumollard and Sardet, 2001 for details) separated by ER-poor domains containing yolk platelets (Fig. 3B, Dumollard and Sardet, 2001). In this layer, the Ca²⁺ wave has a heterogeneous propagation: it spreads quickly in the ER-rich domains (regions 2 and 4 in Fig. 3Bb,c) and slows in the ER-poor domains (regions 3 in Fig. 3Bb,c). Ca²⁺ waves propagate through the whole egg but on a microscopic level, the properties of propagation will be different as the wave passes through distinct cytoplasmic domains and microdomains containing different densities of Ca²⁺ releasing organelles. It has also been observed that, in ascidians, Ca²⁺ waves propagate faster in the cortex than in the egg interior (McDougall and Sardet, 1995). This can be due either to the presence of a higher density of ER-rich domains or to higher amounts of IP₃ in the cortex, or both.

4. Three Ca²⁺ wave pacemakers in a single cell

Three Ca²⁺ wave pacemakers associated with three different cortical ER-rich domains can be observed in the ascidian egg.

The initial pacemaker associated with fertilisation is characterised by a large (7 μM in *Phallusia mammillata*, 10 μM in *Ciona intestinalis*, Speksnijder et al., 1989) and sustained Ca²⁺ wave lasting 3 to 5 minutes in all ascidian species studied (Fig. 2). This Ca²⁺ wave is immediately followed by a series of 1 to 4 repetitive Ca²⁺ waves (1 to 4 μM amplitude in *Phallusia mammillata*, *Asciidiella aspersa*, *Ciona intestinalis* and *Ciona savignyi*) which end at the time of meiosis I completion signalled by the extrusion of the first polar body. After a pause (lasting 2 to 4 minutes in *Phallusia* (Speksnijder et al., 1989), 4 to 6 minutes in *Asciidiella* (McDougall et al., 2000b), 5 minutes in *Ciona intestinalis* (Russo et al., 1996), 6 minutes in *Ciona savignyi* (Kyojuka et al., 1998)), a second series of Ca²⁺ waves is elicited (6 to 15 waves in *Phallusia*, an average of 6 waves in *Asciidiella* (range : 3 to 14 waves, McDougall et al., 2000b) and an average of 14.5±4.2 waves in *Ciona savignyi* (Kyojuka et al., 1998), Fig. 2). This second series is composed of waves of increasing then decreasing amplitude and ceases just before meiosis II ends and a second polar body is emitted

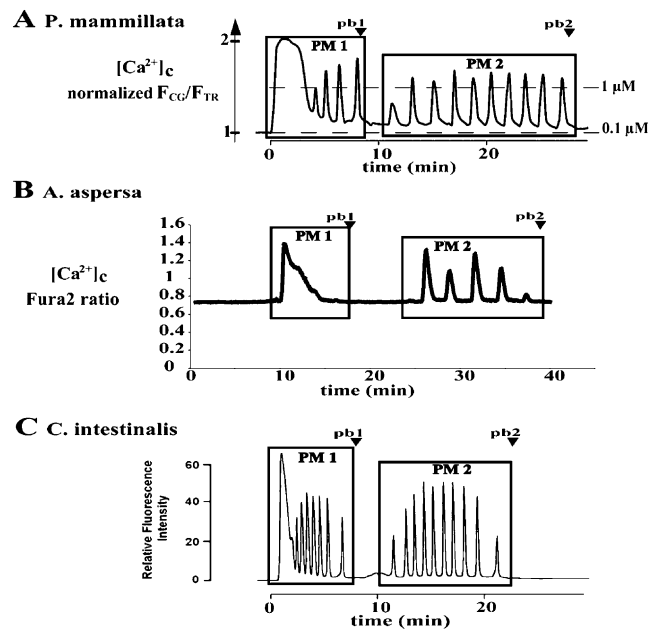


Fig. 2. Sperm-triggered Ca²⁺ oscillations in ascidian eggs.

In eggs of *Phallusia mammillata* (A), *Asciidiella aspersa* (B) and *Ciona intestinalis* (C), sperm entry triggers two series of Ca²⁺ waves initiated by two Ca²⁺ wave pacemakers (PM1 and PM2). The first series of Ca²⁺ wave is composed of a long lasting Ca²⁺ wave of large amplitude followed by repetitive Ca²⁺ waves which lead to the extrusion of the first polar body (pb1). After a pause lasting 2 to 4 minutes in *Phallusia*, 4 to 6 minutes in *Asciidiella* and 6 minutes in *Ciona*, the second series of Ca²⁺ waves is triggered. It lasts 15 to 20 minutes and comprises 6 to 15 waves in *Phallusia*, 3 to 14 waves in *Asciidiella* and an average of 14.5 waves in *Ciona*. This second series ends when the second polar body is emitted (pb2). The values for [Ca²⁺]_c displayed on the right of the graph of *P. mammillata* egg are from aequorin measurements (Speksnijder et al., 1989).

(Fig. 2). The first Ca^{2+} wave pacemaker (PM1) is mobile and relocates from the point of sperm entry (generally in the animal hemisphere) to the vegetal hemisphere while the second pacemaker (PM2) is stably established in the vegetal contraction pole (*Phallusia*: McDougall and Sardet, 1995, *Asciidiella*: McDougall et al., 2000b).

Raising IP_3 uniformly throughout the egg induces an artificial pacemaker (PM3) which lies in the region of the egg that is most sensitive to IP_3 . Surprisingly this artificial pacemaker is always located in the animal hemisphere where the ER-rich meiotic spindle lies. The temporal characteristics of this artificial pacemaker resemble that of the natural meiosis I pacemaker (PM1) with a long lasting Ca^{2+} increase followed by Ca^{2+} oscillations (Dumollard and Sardet, 2001; Dumollard et al., 2002). However this artificial pacemaker is different from PM1 as it is stably established in the animal pole region and is not dislodged by the cortical contractions occurring after each Ca^{2+} wave. In addition, even though this artificial pacemaker can entrain the completion of meiosis I and II it is located opposite to the natural PM2 (always situated in the vegetal hemisphere). This implies that the two naturally occurring pacemakers do not simply rely on the egg's response to a global increase in IP_3 but that they necessitate other factors brought by the fertilising sperm.

At fertilisation sperm injects a sperm factor into the egg that, by itself, induces the two meiotic pacemakers of ascidians (Kyojuka et al., 1998; McDougall et al., 2000a) and the pacemaker of mice (Swann and Parrington, 1999). A candidate for the sperm factor has been recently identified in mammals as a novel form of phospholipase C (PLC) called $\text{PLC}\zeta$ (Saunders et al., 2002; Cox et al., 2002). The sole injection of an mRNA coding for $\text{PLC}\zeta$ in a mouse egg is able to trigger a Ca^{2+} wave pacemaker that is identical to the one induced at fertilisation. The ascidian sperm factor seems however different even though its nature has not been elucidated yet. It may be a $\text{PLC}\gamma$ or an activator of $\text{PLC}\gamma$ (Runft and Jaffe, 2000). Since this topic has been discussed extensively it will not be detailed here (Swann and Parrington, 1999; Nixon et al., 2000; McDougall et al., 2000a; McDougall et al., 2000b; Runft et al., 2002; Dumollard et al., 2002).

In ascidians the sperm also transmits its basal body to the egg and this is at the origin of the growth of a sperm aster in the cortex which recruits ER within minutes of fertilisation. The fertilisation Ca^{2+} wave starts from the point of sperm entry and subsequent waves emitted by the moving PM1 are initiated from the ER-rich region forming around the sperm aster (Dumollard and Sardet, 2001). This strongly suggests that the sperm factor stays in the cortex of the egg and is dragged with the material introduced by the sperm towards the vegetal pole as a consequence of the microfilament-driven cortical contraction. The temporal pattern of this first pacemaker (PM1) can be artificially reproduced by generating a long-lasting increase in IP_3 (Dumollard and Sardet, 2001) suggesting that the sperm factor produces a long-lasting increase of IP_3 while it is relocated towards the vegetal pole. During the gap time between the two series of

Ca^{2+} waves successively triggered by PM1 and PM2 IP_3 production apparently ceases transiently (McDougall and Levasseur, 1998). Upon entry into meiosis II PM2 initiates the second series of Ca^{2+} waves from another accumulation of cortical ER lying in the vegetal contraction pole (McDougall and Sardet, 1995; Dumollard and Sardet, 2001; McDougall et al., 2000b). The contraction pole is also rich in PIP_2 since it contains an accumulation of plasma membrane in the form of dense microvilli (Roegiers et al., 1995; Carroll et al., 2003). However eliminating this tuft of microvilli with the microfilament inhibitor cytochalasin does not alter the ability of PM2 to emit waves suggesting that the local enrichment of PIP_2 is completely dispensable for the operation of PM2 (Carroll et al., 2003). Ca^{2+} influx is also unnecessary for this pacemaker to function (Speksnijder et al., 1989; Carroll et al., 2003). Therefore it seems that the main factor that determines the site of PM2 is a local source of IP_3 production in the contraction pole (likely provided by the sperm factor).

The two natural pacemakers of the ascidian egg appear to be made of a local source of IP_3 apposed to a cortical ER-rich domain with the local source of IP_3 being the main factor that determines the pacemaker site. Imaging the IP_3 production in the egg or monitoring the sperm factor in the fertilised egg is required to confirm these hypotheses. They are nonetheless the most parsimonious to explain the occurrence of the two physiological Ca^{2+} wave pacemakers with stereotyped spatial characteristics in a single ascidian egg.

5. What roles for cortical Ca^{2+} wave pacemakers?

The functional and developmental significance of cortical pacemakers is presently a matter of speculation. PM1 the first pacemaker of the ascidian zygote is associated with the sperm aster which, after duplication takes place (soon after meiosis completion), will define the first cleavage plane and the future posterior pole of the embryo. PM2 lies in the vegetal contraction pole where determinants concentrate (maternal determinants for muscle, endoderm formation and morphogenetic determinants (unequal division, gastrulation), Nishida, 1997). The determinants as well as numerous other maternal mRNAs (called PEM or postplasmic RNAs, Sasakura et al., 2000) are contained in the contraction pole and they will experience the largest variations in $[\text{Ca}^{2+}]_c$ setting up a periodic Ca^{2+} gradient highest in the vegetal hemisphere. It is remarkable that the recently identified muscle determinant (macho1 mRNA, Nishida and Sawada, 2001) is in fact located on the ER which accumulates in the contraction pole (Sardet et al., 2003). The close proximity of these determinants with the origin of the meiosis II Ca^{2+} waves suggests that some Ca^{2+} dependant processes may play a role in the maturation or priming of these determinants.

The mitochondria-rich domain located in the vegetal sub-cortex (Fig. 1) and apposed to the interior face of the contraction pole segregates in the muscle cells of the embryo (Jef-

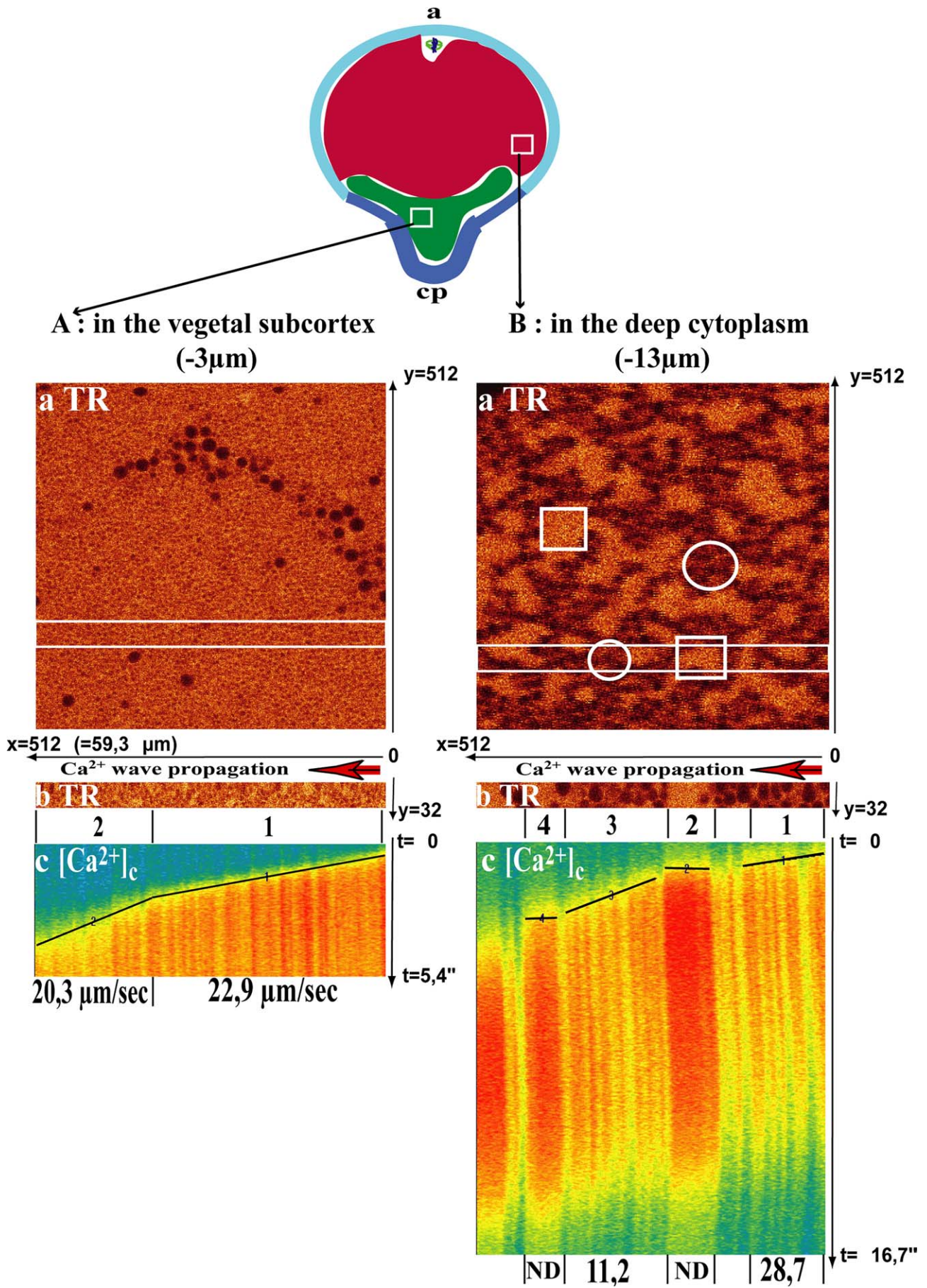


Fig. 3. High speed imaging (1 image/37 msec) of meiotic Ca^{2+} waves propagation in the different cytoplasmic domains of the ascidian egg.

A,B Confocal imaging of two Ca^{2+} waves emitted by PM2. In A the wave propagates in a 7 μm -thick domain situated about 3 μm beneath the egg surface. In B the wave propagates in the deeper cytoplasm (13 μm beneath the egg surface) made of ER-rich and yolk-rich microdomains.

(a) Texas Red Dextran (TR) images of the vegetal subcortex (512X512 pixels) showing the punctate mitochondria-rich domain (A) and deeper cytoplasm (B) with ER-rich (one of which is indicated by a square) and yolk-rich microdomains (one of which is indicated by a circle). The image has been tilted by 90° compared to schemes and the vegetal pole is on the right of the image whereas the animal pole is on the left.

(b) TR image obtained during high speed imaging of the Ca^{2+} wave (one image 512X32 pixels every 37 msec). The image corresponds to the region indicated by white horizontal lines in a.

(c) temporal image of propagating Ca^{2+} waves emitted by PM2 in the mitochondria-rich subcortex (A) or in the deeper cytoplasm (B). The vertical axis of the image display the time elapsed (t). The $[\text{Ca}^{2+}]_c$ is measured from a ratiometric imaging of 512X32 pixels region (Calcium Green divided by Texas Red: blue indicates lower $[\text{Ca}^{2+}]_c$ and red higher $[\text{Ca}^{2+}]_c$, see Dumollard and Sardet 2001 for details). Each 512X32 pixels ratiometric image has been averaged along the y axis to give a “line image”. All the line images were then stacked together to make the temporal interpreted from the slope of the propagation front of the Ca^{2+} wave.

In (A), the wave propagates homogeneously in the mitochondria-rich domain (region 1) before slowing down (region 2). In the deeper cytoplasm (B), the wave shows a heterogeneous propagation, accelerating in the ER-rich domains (regions 2, 4) and slowing down in the yolk-rich domains (region 3). The wave also slows down as it travels further into the deeper cytoplasm. The speed of the wave in the different regions is indicated. N. D.: not determined.

fery and Swalla, 1990). The mitochondria in this subcortical domain will sense and decode the Ca^{2+} oscillations and one can imagine that these mitochondria are primed by the Ca^{2+} waves before the onset of myogenesis in the embryo.

Interestingly in nemertean whose eggs also display Ca^{2+} oscillations (Stricker, 1999), the Ca^{2+} waves all come from the vegetal pole which, as in ascidians, becomes the site of gastrulation of the embryo. In these embryos, the dorso-ventral axis of the embryo is defined at the first cleavage (Roegiers et al., 1995; Henry and Martindale, 1996) we may wonder whether the vegetal Ca^{2+} wave pacemaker participates in axis establishment.

There is some evidence that repetitive Ca^{2+} signals are required for mammalian development: experiments with rabbit eggs artificially activated by particular regimes of electrical pulses that induce repetitive Ca^{2+} transients show that the ability of the embryo to cleave and develop in utero for several days after reimplantation is dependant on the frequency and amplitude of the repetitive Ca^{2+} transients generated by the electrical pulses (Ozil and Huneau, 2001). Resolving the question of the role of vegetal Ca^{2+} wave pacemakers in development will require manipulation of the normal spatio-temporal pattern of meiotic Ca^{2+} waves. This can now be achieved in the ascidian embryo by inhibiting the natural PM2 and by replacing it by the artificial PM3. If different phenotypes can be associated with different Ca^{2+} wave patterns this would establish for the first time a role for polarised Ca^{2+} signals in development.

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References

- Albrieux, M., Sardet, C., Villaz, M., 1997. The two intracellular Ca^{2+} release channels ryanodine receptor and inositol 1,4,5-triphosphate receptor, play different roles during fertilisation in ascidians. *Dev. Biol.* 189, 174–185.
- Albrieux, M., Lee, H. C., Villaz, M., 1998. Calcium signalling by cyclic ADP-ribose, NAADP, and inositol trisphosphate are involved in distinct functions in ascidian oocytes. *J Biol Chem* 273, 14566–14574.
- Albrieux, M., Moutin, M. J., Grunwald, D., Villaz, M., 2000. Calmodulin and immunophilin are required as functional partners of a ryanodine receptor in ascidian oocytes at fertilisation. *Dev. Biol.* 225, 101–111.
- Arnoult, C., Grunwald, D., Villaz, M., 1996. Novel postfertilisation inward Ca^{2+} current in ascidian eggs ensuring a calcium entry throughout meiosis. *Dev. Biol.* 174, 322–334.
- Carroll, J., 2001. The initiation and regulation of Ca^{2+} signalling at fertilisation in mammals. *Sem. Cell and Dev. Biol.* 12, 37–43.
- Carroll, M., Levasseur, M., Jones, K.T., Wood, C., Whitaker, M., McDougall, A., 2003. Calcium wave pacemaker in fertilized ascidian eggs submitted.
- Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K., Lai, F.A., 2002. Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction.* 124 (5), 611–623.
- Deguchi, R., Shirakawa, H., Oda, S., Mohri, T., Miyazaki, S., 2000. Spatiotemporal analysis of Ca^{2+} waves in relation to the sperm entry site and animal-vegetal axis during Ca^{2+} oscillations in the fertilized mouse eggs. *Dev. Biol.* 218, 299–313.
- Duchen, M., 2000. Mitochondria and calcium: from cell signalling to cell death. *J. Physiol.* 529.1, 57–68.
- Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R.M., Kopf, G.S., Fissore, R., Madoux, S., Ozil, J.P., 2002. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev. Biol.* 250 (2), 280–291.
- Dumollard, R., Sardet, C., 2001. Three different calcium wave pacemakers in ascidian eggs. *J. Cell Sci.* 114, 2471–2481.
- Dumollard, R., Carroll, J., Dupont, G., Sardet, C., 2002. Calcium wave pacemakers in eggs. *J. Cell Sci.* 115, 3557–3564.
- Dumollard, R., Hammar, K., Porterfield, M., Smith, P.J., Cibert, C., Rouviere, C., Sardet, C., 2003. Mitochondrial respiration and Ca^{2+} waves are linked during fertilisation and meiosis completion. *Development.* 130 (4), 683–692.
- Goudeau, M., Goudeau, H., 1993. In the egg of the ascidian *Phallusia mammillata*, removal of external Ca^{2+} modifies the fertilisation potential, induces polyspermy, and blocks the resumption of meiosis. *Dev. Biol.* 160, 165–177.

- Henry, J. Q., Martindale, M. Q., 1996. The establishment of embryonic axial properties in the nemertean, *Cerebratulus lacteus*. *Dev. Biol.* 180, 713–721.
- Jeffery, W.R., Swalla, B.J., 1990. The myoplasm of ascidian eggs: a localized cytoskeletal domain with multiple roles in embryonic development. *Semin. Cell Biol.* 1 (5), 373–381.
- Kondoh, M., Kasai, T., Shimada, M., Kashiwayanagi, M., Yokosawa, H., 2003. cDNA cloning and characterization of an osmotically sensitive TRP channel from ascidian eggs. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 134 (3), 417–423.
- Kyozuka, K., Deguchi, R., Mohri, T., Miyazaki, S., 1998. Injection of sperm extract mimics spatiotemporal dynamics of Ca²⁺ responses and progression of meiosis at fertilisation of ascidian oocytes. *Development* 125, 4099–4105.
- McDougall, A., Sardet, C., 1995. Function and characteristics of repetitive calcium waves associated with meiosis. *Curr. Biol.* 5, 318–328.
- McDougall, A., Levasseur, M., 1998. Sperm-triggered calcium oscillations during meiosis in ascidian oocytes first pause, restart, then stop: correlations with cell cycle kinase activity. *Development* 125 (22), 4451–4459.
- McDougall, A., Levasseur, M., O'Sullivan, A. J., Jones, K. T., 2000a. Cell cycle-dependent repetitive Ca(2+) waves induced by a cytosolic sperm extract in mature ascidian eggs mimic those observed at fertilisation. *J. Cell Sci.* 113, 3453–3462.
- McDougall, A., Shearer, J., Whitaker, M., 2000b. The initiation and propagation of the fertilisation wave in sea urchin eggs. *Biol. Cell* 92, 205–214.
- Nishida, H., 1997. Cell fate specification by localized cytoplasmic determinants and cell interactions in ascidian embryos. *Int. Rev. Cytol.* 176, 245–306.
- Nishida, H., Sawada, K., 2001. macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. *Nature* 409, 724–729.
- Nixon, V. L., McDougall, A., Jones, K. T., 2000. Calcium oscillations and the cell cycle at fertilisation of mammalian and ascidian eggs. *Biol. Cell* 92, 187–196.
- Ozil, J.P., Huneau, D., 2001. Activation of rabbit oocytes: the impact of the Ca²⁺ signal regime on development. *Development* 128 (6), 917–928.
- Rizzuto, R., Bernardi, P., Pozzan, T., 2000. Mitochondria as all-round players of the calcium game. *J. Physiol.* 529.1, 37–47.
- Roegiers, F., McDougall, A., Sardet, C., 1995. The sperm entry point defines the orientation of the calcium-induced contraction wave that directs the first phase of cytoplasmic reorganization in the ascidian egg. *Development* 121 (10), 3457–3466.
- Roegiers, F., Djediat, C., Dumollard, R., Rouviere, C., Sardet, C., 1999. Phases of cytoplasmic and cortical reorganizations of the ascidian zygote between fertilisation and first division. *Development* 126, 3101–3117.
- Runft, L. L., Jaffe, L. F., 2000. Sperm extract injection into ascidian eggs signals Ca²⁺ release by the same pathway as fertilisation. *Development* 127, 3227–3236.
- Runft, L.L., Jaffe, L.A., Mehlmann, L.M., 2002. Egg activation at fertilisation: where it all begins. *Dev. Biol.* 245 (2), 237–254.
- Russo, G.L., Kyozuka, K., Antonazzo, L., Tosti, E., Dale, B., 1996. Maturation promoting factor in ascidian oocytes is regulated by different intracellular signals at meiosis I and II. *Development* 122 (7), 1995–2003.
- Sardet, C., Speksnijder, J., Terasaki, M., Chang, P., 1992. Polarity of the ascidian egg cortex before fertilisation. *Development* 115 (1), 221–237.
- Sardet, C., Roegiers, F., Dumollard, R., Rouviere, C., McDougall, A., 1998. Calcium waves and oscillations in eggs. *Biophysical Chem.* 72, 131–140.
- Sardet, C., Prodon, F., Dumollard, R., Chenevert, J., Chang, P., 2002. Structure and function of the egg cortex. *Dev. Biol.* 241 (1), 1–23.
- Sardet, C., Nishida, H., Prodon, F., Sawada, K., 2003. Maternal mRNAs of PEM and macho-1, the ascidian muscle determinant, associate and move with a rough endoplasmic reticulum network in the egg cortex. *Development*. in press.
- Sasakura, Y., Ogasawara, M., Makabe, K.W., 2000. Two pathways of maternal RNA localization at the posterior-vegetal cytoplasm in early ascidian embryos. *Dev. Biol.* 220 (2), 365–378.
- Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 129 (15), 3533–3544.
- Sensui, N., Morisawa, M., 1996. Effect of Ca²⁺ on deformation, polar body extrusion and pronucleus formation in the egg of the ascidian, *Ciona savignyi*. *Dev. Growth Differ.* 38, 341–350.
- Speksnijder, J., Corson, D., Sardet, C., Jaffe, L., 1989. Free calcium pulses following fertilisation in the ascidian egg. *Dev. Biol.* 135, 182–190.
- Speksnijder, J., Terasaki, M., Hage, W., Jaffe, L., Sardet, C., 1993. Polarity and reorganization of the endoplasmic reticulum during fertilisation and ooplasmic segregation in the ascidian egg. *J. Cell Biol.* 120, 1337–1346.
- Stricker, S., 1999. Comparative biology of calcium signaling during fertilisation and egg activation in animals. *Dev. Biol.* 211, 157–176.
- Swann, K., Parrington, J., 1999. Mechanism of Ca²⁺ release at fertilisation in mammals. *J. Exp. Zool.* 285 (3), 267–275.
- Yoshida, M., Sensui, N., Inoue, T., Morisawa, M., Mikoshiba, K., 1998. Role of two series of Ca²⁺ oscillations in activation of ascidian eggs. *Dev. Biol.* 203, 122–133.