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Review

Calcium signals and mitochondria at fertilisation

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Abstract

At fertilisation, Ca^{2+} signals activate embryonic development by stimulating metabolism, exocytosis and endocytosis, cytoskeletal remodelling, meiotic resumption and recruitment of maternal RNAs. Mitochondria present in large number in eggs have long been thought to act as a relay in Ca^{2+} signalling at fertilisation. However, only recently have studies on ascidians and mouse proven that sperm-triggered Ca^{2+} waves are transduced into mitochondrial Ca^{2+} signals that stimulate mitochondrial respiration. Mitochondrial Ca^{2+} uptake can substantially buffer cytosolic Ca^{2+} concentration and the concerted action of heterogeneously distributed mitochondria in the mature egg may modulate the spatiotemporal pattern of sperm-triggered Ca^{2+} waves. Regulation of fertilisation Ca^{2+} signals could also be achieved through mitochondrial ATP production and mitochondrial oxidant activity but these hypotheses remain to be explored. A critically poised dynamic interplay between Ca^{2+} signals and mitochondrial metabolism is stimulated at fertilisation and may well determine whether the embryo can proceed further into development. The monitoring of Ca^{2+} signals and mitochondrial activity during fertilisation in living zygotes of diverse species should confirm the universality of the role for sperm-triggered Ca^{2+} waves in the activation of mitochondrial activity at fertilisation.

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Keywords: Fertilisation; Mitochondria; Ca^{2+} oscillations; ATP; Oxidative stress

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1. Introduction

Mitochondria were first described in 1890 as “bioblasts: a cytoplasmic structure of ubiquitous occurrence, resembling bacteria and functioning as elementary organisms” [1]. Much later, this idea was confirmed by the discovery that mitochondria possess their own DNA, a unique nucleic acid coding sequence system and that they can replicate independently of the nuclear cell cycle. Mitochondria contain the enzymes of the citric cycle, fatty acid oxidation and oxidative phosphorylation making them the major site for production of ATP in eukaryotic cells [1].

Abbreviations: $[\text{Ca}^{2+}]_{\text{cyto}}$, cytosolic Ca^{2+} concentration; $[\text{Ca}^{2+}]_{\text{mito}}$, mitochondrial Ca^{2+} concentration; ATP, adenosine triphosphate; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; GV, germinal vesicle; ER, endoplasmic reticulum; IP3, inositol 3,4,5 trisphosphate; IICR, IP3-induced Ca^{2+} release; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; PM1, pacemaker 1; PM2, pacemaker 2; SERCA, sarco-endoplasmic Ca^{2+} ATPase; PMCA, plasma membrane Ca^{2+} ATPase; GFP, green fluorescent protein; O_2 , oxygen; O_2^- , superoxide; H_2O_2 , hydrogen peroxide; CN^- , cyanide

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Besides the housekeeping roles of mitochondria in the cell, more specific roles were found recently in somatic cells, notably in intracellular Ca^{2+} signalling and in the apoptotic cascade [2,3].

One peculiarity of mitochondria in development is that they are transmitted to the next generation exclusively from the mother and independently from the nuclear genome [4–6,28]. An enormous number of mitochondria (between tens of thousands and tens of millions depending on the species) is produced during oogenesis from a restricted founder population present in the primordial germ cell. This mechanism provides a genetic bottleneck for transmission of mitochondrial DNA (mtDNA) which ensures that there is a uniform population of mitochondria in the mature oocyte [5,6,24]. This uniformity of the mitochondrial population is primordial for the early embryo as mitochondrial replication does not resume during cleavage stage and the mitochondria present in the oocyte are dispatched between the different blastomeres of the cleaving embryo.

In oocytes and zygotes of many organisms (amphibians, fishes, insects, planarians, chaetognaths, nematodes and some mammals) two populations of mitochondria can be found: one population is aggregated in a specialised structure in the egg cytoplasm called the germ plasm (or nuage or Balbiani body or pole plasm) that segregates to the germ line and is necessary to specify the germ line [7]. The rest of the mitochondria are scattered in the whole of the oocyte and transmitted to the somatic lineage. Apart from their replicative activity, it is not known whether the mitochondria of these two populations are functionally distinct. Yet, criteria that define which mitochondria will associate with the germ plasm are emerging [7,8].

A paradigm introduced by electron microscopists in the 60s suggests that mitochondria in oocytes are immature as they possess only few cristae compared to the numerous cristae and electron dense material found in mitochondria of post-implantation embryos and actively respiring cells [9,10] and references within). This led to the idea that mitochondria are minimally active in the oocyte and hence play a negligible part in the activation of development. Nevertheless due to their enormous number, even with each mitochondrion having a low activity, the concerted contribution of all the egg's mitochondria to the egg physiology is major while, at the same time, a low metabolism of individual mitochondria minimises oxidative stress [11,12]. Recent observations strongly suggest that mammalian mature oocytes display a high ATP turnover and that the ATP consumed is supplied by mitochondrial respiration [13,14]. Furthermore, a critical role for active mitochondria in the regulation of sperm-triggered Ca^{2+} waves was demonstrated in ascidian [15] and mouse zygotes [13,16].

One of the most ubiquitous features of fertilisation is the triggering by sperm entry of a single or repetitive Ca^{2+} waves (reviewed by Miyasaki, in this issue). The first direct measurement of the mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mito}}$) in living somatic cells established more than 10 years ago that mitochondria can sequester cytosolic Ca^{2+} and tune their metabolic activity to the pattern of cytosolic Ca^{2+} signals [17–19]. However, in eggs, Ca^{2+} sequestering by mitochondria at fertilisation has not been fully characterised and the stimulation of mitochondrial activity by sperm-triggered Ca^{2+} waves has only

been demonstrated recently in ascidian and mouse [13,15]. The dynamic interplay between Ca^{2+} signalling and mitochondrial activity provides several mechanisms to subtly modulate the fertilisation Ca^{2+} waves. Three functions of mitochondria could significantly influence the spatio-temporal pattern of sperm-triggered Ca^{2+} waves: buffering of cytosolic Ca^{2+} , production of ATP and production of reactive oxygen species (ROS). The first two functions of mitochondria have been clearly demonstrated in zygotes [13,15,16] and several observations suggest that the third function may occur in mammalian and sea urchin zygotes [20,21,23].

This review will describe how mitochondria produced during oogenesis are stimulated by sperm-triggered Ca^{2+} signals and how, in return, they modulate these Ca^{2+} signals thus regulating egg activation. The more specific question of mitochondrial inheritance has been reviewed elsewhere [6,27,28]. The reader interested in broader implications of mitochondria during more general processes of development can refer to other reviews [24,25,29,30–32].

2. Mitochondrial biogenesis and inheritance in the oocyte

The egg cell possesses a large number of mitochondria. It is both due to the large size of the egg and to the fact that oocytes store a finite number of mitochondria that does not need to be increased until the onset of embryonic growth. Estimates of mitochondrial number in eggs obtained from analysis of electron microscopy or of mtDNA copy number (with an estimate of 1–2 mtDNA copies per mitochondrion) showed that the small *C. elegans* egg possesses the smallest number of mitochondria (18,000 mtDNA copies [33]), followed by sea urchin (between 150,000 and 300,000 mitochondria [34]), mammals (from 150,000 to 800,000 mtDNA copies [27,24], *Xenopus* (10,000,000 mitochondria [35]), *Drosophila* (15,000,000 mtDNA copies [99]) and fishes (70–200 million mtDNA copies [36]).

Mitochondrial replication starts during the previtellogenic phase of oogenesis and ceases at the end of maturation (mammals [24,27]; sea urchin [37]; *Xenopus* [38]; fish [36]; *C. elegans* [32,33]). During vitellogenesis, an enormous amplification of a founding population of mitochondria occurs (from only 10 mitochondria per human primordial germ cell to several hundred thousands in a mature oocyte [27]). This amplification of germ cell mitochondria explains why a high degree of homoplasmy of mtDNA is found in mature eggs even though mitochondrial DNA is prone to mutations [6,39]. Indeed, as all the mitochondria derive from a limited number of precursors, they all have the same genetic origin and probably the same metabolic potential. The presence of a homogeneous population of mitochondria in the oocyte is very important to maintain an equivalent viability of each blastomere which will receive only a portion of the oocyte's mitochondria. Mitochondrial replication does not resume before gastrulation in fishes [36], before the swimming tadpole stage in *Xenopus* [38], before implantation in mammals [26] or larval stage in *C. elegans* [33]. Moreover, in the mouse, development until blastocyst stage is not altered by

inhibiting either mitochondrial DNA replication or mitochondrial translation [40,41,42] further demonstrating that the mitochondrial population stored in the oocyte is able to support early development.

As stated before, a subpopulation of mitochondria associated with the germ plasm that will segregate to the germ line can be distinguished in oocytes of several species. This subset of mitochondria is observable from the first stage of oogenesis in *C. elegans*, *Xenopus*, insects, chicken and goat as a mitochondrial cloud located in the vicinity of the germinal vesicle (Fig. 1D

and E [7]). During the initial stages of oogenesis in *Xenopus*, the mitochondrial cloud is a site of active mitochondrial replication [43]. Then the mitochondrial cloud fragments and two segregating populations of mitochondria arise. One population remains around the nucleus, actively replicating mtDNA that ultimately generates the majority of the mitochondria in the fully grown oocyte [43]. The other population of mitochondria destined to the germ line migrates towards the vegetal cortex (together with germ plasm and germ line determinants) and stops replicating early in vitellogenesis [7,43,48]. Apart from their replicating

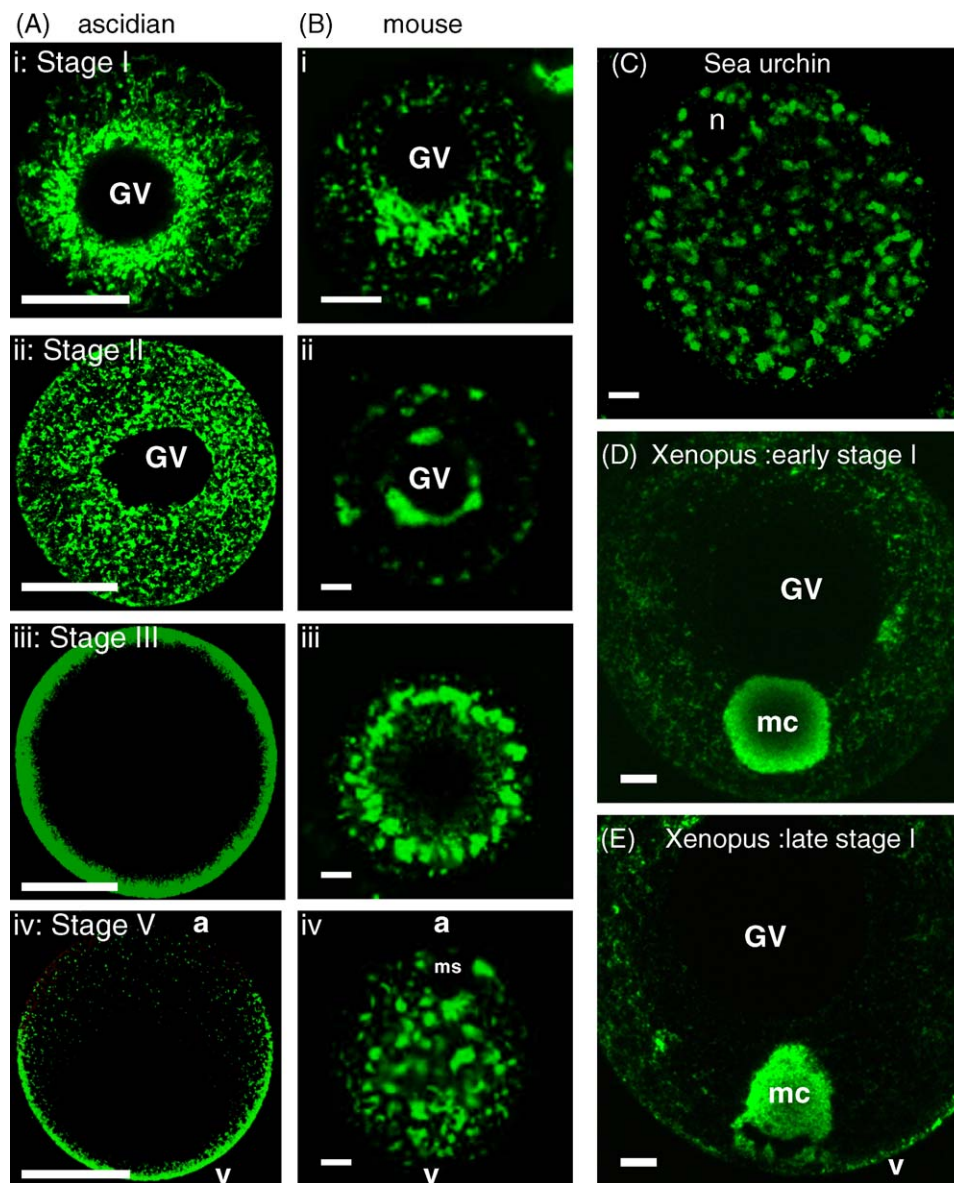


Fig. 1. Distribution of mitochondria in eggs. Mitochondria were stained with the mitochondrial dye TMRE [2,13,15,19] in oocytes of different species. (A) Mitochondrial distribution during oogenesis in the ascidian: (i) stage I previtellogenic oocyte (scale bar: 15 μm); (ii) stage II early vitellogenic oocyte (scale bar: 25 μm); (iii) stage III late vitellogenic oocyte (scale bar: 35 μm); and (iv) stage IV mature metaphase I-arrested oocyte (scale bar: 50 μm). GV, germinal vesicle; a, animal pole; v, vegetal pole (see [49]). (B) Mitochondrial distribution during oogenesis in the mouse: (i) meiotically incompetent oocyte (scale bar: 10 μm); (ii) fully grown GV stage oocyte (scale bar: 10 μm); (iii) maturing oocyte just after GVBD (scale bar: 10 μm); and (iv) mature metaphase II-arrested oocyte (scale bar: 10 μm). ms, Meiotic spindle; a, animal pole; v, vegetal pole (C) Mature sea urchin oocyte (scale bar: 10 μm). n, Nucleus. (D) *Xenopus*: mitochondrial cloud (mc) located next to the GV of an early stage I oocyte. Scale bar: 23 μm . (E) *Xenopus*: mitochondrial cloud (mc) starting to fragment upon reaching the vegetal (v) cortex of a late stage I oocyte. Scale bar: 23 μm .

activity it is not yet known whether the mitochondria of the germ plasm have different activity than the mitochondria in the rest of the egg (but see [44]).

Drosophila eggs display an extreme example of segregation of germ plasm mitochondria: during oogenesis, germ plasm mitochondria are transported from the nurse cells into the growing oocyte on a specialised structure called the fusome [8,45]. This fusome and associated mitochondria travel through intercellular bridges between nurse cells and the oocyte called ring canals [45]. After the fusome has entered the oocyte, the other mitochondria from the nurse cells seem blocked at the entry of the ring canals suggesting that their passage into the oocyte becomes restricted [8]. Approximately 1/16th of the mitochondrial population of the mature oocyte arises directly in the oocyte itself but it remains to be established whether these mitochondria can mix with the mitochondria of the germ plasm (at the posterior pole of the egg) or whether they are excluded from the posterior pole of the egg at the end of maturation. Ring canals have also been observed between germ cells in *Xenopus* and mouse but their much smaller size may not allow efficient passage of mitochondria [7,46,47] suggesting that, in these species, most (if not all) of the mitochondria of the oocyte arise in the oocyte itself. If the origin of the founding population of mitochondria in the egg has been well traced, further studies are required to understand the regulation of mitochondrial amplification during oogenesis and mitochondrial maintenance during early development.

3. Mitochondrial organisation, distribution and shapes in oocytes

During maturation, the period during which the oocyte acquires its ability to respond to the fertilising sperm by eliciting Ca^{2+} signals, the growing population of mitochondria is often relocated in different regions of the oocyte. It seems that the early phases of mitochondrial generation show strong similarities between species whereas the final phases of mitochondrial relocation during maturation are more divergent and generate the very variable patterns of mitochondrial distribution observed in mature oocytes. Before maturation the bulk of mitochondria is located in the vicinity of the large nucleus (called the germinal vesicle: GV) where they first concentrate in one or several clusters (*Xenopus* [48]; mouse, Fig. 1Bi [47]; human [6]). In GV stage oocyte mitochondria surround the GV (ascidian, Fig. 1Aii [49]; *Xenopus* [48], mammals, Fig. 1Bii [50,53]) and then migrate from the centre to the periphery of the oocyte after GV breakdown (GVBD, Fig. 1 [48–50,53]).

In *Xenopus*, the mitochondrial cloud assembling around the GV migrates away from the GV to populate the cytoplasm and line up against the vegetal cortex [7,48]). A population of cortical mitochondria can be observed in the immature oocyte (Fig. 1E [51]) but the bulk of mitochondria are in the central cytoplasm in the mature oocyte [52].

In the mouse, mitochondria first accumulated around the GV in fully grown GV stage oocyte (Fig. 1Bii), move away from the peri-nuclear region during formation and migration of the first meiotic spindle towards the animal pole (GVBD,

Fig. 1Biii [53]). In the mature metaphase II-arrested oocyte, mitochondria are enriched in the centre of the egg and around the meiotic spindle and are scarcer in the vegetal cortex (Fig. 1Biv [13,54]).

In the ascidian *Ciona intestinalis*, the vast majority of mitochondria are concentrated in a ring around the oocyte's GV during previtellogenic stages (stage I, Fig. 1Ai). Early vitellogenic stages oocytes (stage II) are characterised by an abundance of mitochondria distributed more homogeneously in the cytoplasm and the cortex (Fig. 1Aii). At late vitellogenic stages (stage III), mitochondria are found mostly at the cell periphery, forming a 7–10 μm -thick subcortical domain (Fig. 1Aiii). At this stage, this peripheral ring of mitochondria is symmetric, with no indication of the presence of animal or vegetal poles [49]. In oocytes which have completed maturation (stage IV), the subcortical layer of mitochondria (called myoplasm) are excluded from the animal pole region and form a dense 7 μm -thick subcortical layer lining the vegetal and equatorial regions of the oocyte (Fig. 1Aiv [15,55–57]).

In *Drosophila* and *Xenopus* eggs some mitochondria associate with germ plasm containing mRNA determinants (*Oskar*, *Xcat2*), localising in the posterior or vegetal cortex of the mature egg underlying the polarity of the egg [7,8,51]. In two sea urchin species (*L. pictus* and *Strongylocentrotus purpuratus*), a gradient of mitochondrial density has recently been described in the mature oocyte [58] but the presence of such a gradient has not been observed in earlier studies and in other sea urchin species ([34], Fig. 1C). Therefore, in eggs of a variety of species there exist polar differences in mitochondrial density in the mature oocyte that may well be a maternal factor impacting later development of the embryo. However, it has been shown only in ascidians and sea urchins that this inhomogeneous distribution of mitochondria effectively influences the patterning of the embryo [58,98].

Mitochondria in mature eggs are either rod-shaped (*Xenopus* [51,59]; ascidian [15]) or more oblong or spherical (mouse [13]; pig [50]; human [60]) whereas sea urchin eggs have both rod-shaped and spherical mitochondria [34]. Changes in mitochondrial structure have been reported during oogenesis in humans where mitochondrial cristae are forming before maturation suggesting that mitochondria in the developing oocyte gradually acquire their capacity to generate ATP [60].

From early on during maturation, mitochondria are found in clusters in close proximity to endoplasmic reticulum (ER) membranes (mammals [6,13,47]; *Xenopus* [7,44,51]; ascidian [15]; sea urchin [34]). Such close proximity creates a local microenvironment in the immediate vicinity of ER and mitochondria which experiences much larger variations of soluble metabolites than bulk cytoplasm. For example, as mitochondria are a source of ATP and ROS and ER is the major site of intracellular Ca^{2+} release, the concentrations of ATP, Ca^{2+} and ROS in the microdomain formed between juxtaposed ER and mitochondria will exceed by far the levels of these products measured in the bulk cytosol. This juxtaposition of mitochondria and ER supports the idea that privileged functional interactions exist between the two organelles allowing, among others, an efficient transmission of cytosolic Ca^{2+} signals into the mitochondria

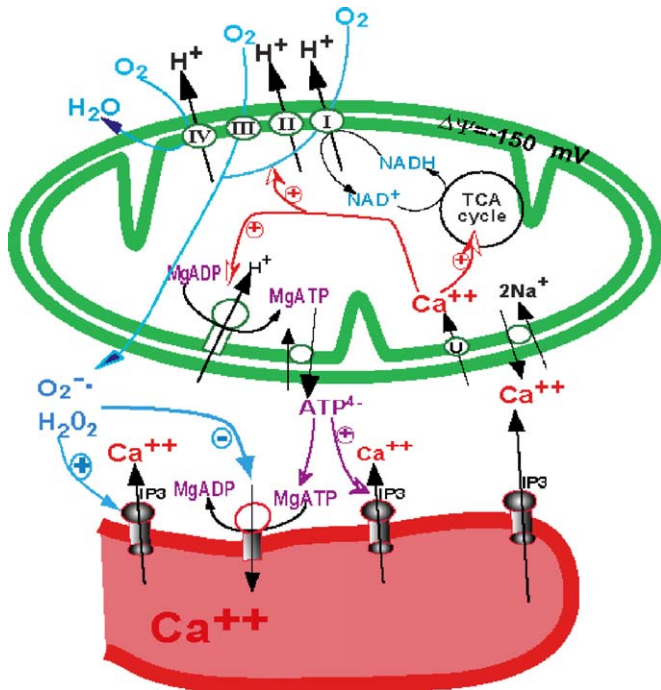


Fig. 2. Functional interactions between mitochondria and ER Ca^{2+} release sites (see [13,15]). Calcium released in the cytosol via IP_3R enters mitochondria via the Ca^{2+} uniporter (U). Mitochondrial Ca^{2+} is then extruded from the mitochondria via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Ca^{2+} in the mitochondrial matrix stimulates the Krebs cycle (TCA cycle which generates NADH), the respiratory chain and the ATP synthase. Activity of the respiratory chain promotes formation of superoxide (O_2^-) at complexes I and III while complete O_2 reduction occurs at complex IV. ATP^{4-} is exported from the mitochondria into the cytosol where it stimulates Ca^{2+} release by sensitizing IP_3Rs to Ca^{2+} while Mg-ATP energizes Ca^{2+} pumping back into the ER lumen and replenish Ca^{2+} stores. Superoxide and H_2O_2 stimulate IP_3R opening and inhibit the Ca^{2+} pumps of the ER (SERCA) thereby enhancing intracellular Ca^{2+} release.

[2,17] and an efficient supply of ATP to the Ca^{2+} pumps of the ER (SERCAs) which replenish the ER Ca^{2+} stores (Fig. 2 [96]).

4. Mitochondria take up Ca^{2+} during the passage sperm-triggered Ca^{2+} waves

Direct and specific measurements of $[\text{Ca}^{2+}]$ in the mitochondrial matrix of living somatic cells ($[\text{Ca}^{2+}]_{\text{mito}}$) have definitively proven that mitochondria can sequester and release substantial amounts of Ca^{2+} during physiological Ca^{2+} signals [2,17,18,92] (see Fig. 2 for a description of the mitochondrial Ca^{2+} influx and efflux pathways). Surprisingly in eggs such measurements of changes in $[\text{Ca}^{2+}]_{\text{mito}}$ during sperm-triggered Ca^{2+} signals have yet to be reported. Nevertheless, indirect arguments based on pharmacological approaches and electron probe microanalysis of total mitochondrial Ca^{2+} suggested for the first time 20 years ago that mitochondria could accumulate Ca^{2+} after the passage of the sperm-triggered Ca^{2+} waves [61,62].

Ca^{2+} sequestering by mitochondria at fertilisation—revealed pharmacologically as an FCCP-releasable Ca^{2+} pool—has been characterised for the first time in the sea urchin egg. Mitochondria have an electronegative potential providing a strong driving force for the influx of Ca^{2+} into their matrix. Col-

lapsing the mitochondrial electrical potential with FCCP will therefore stop Ca^{2+} influx but also release the Ca^{2+} previously sequestered in the mitochondria (see [2,63]). A FCCP-releasable Ca^{2+} pool—absent before fertilisation—can be detected after the end of the fertilisation Ca^{2+} wave in sea urchin eggs [61] and after sperm-triggered Ca^{2+} waves in the ascidian egg [15] strongly suggesting that mitochondria in these eggs accumulate Ca^{2+} during the passage of the Ca^{2+} waves. Electron probe microanalysis of total Ca^{2+} has confirmed that, in sea urchin zygotes, mitochondria accumulate Ca^{2+} at fertilisation [62] but so far these observations have not been extended to other species.

The lack of direct measurements of $[\text{Ca}^{2+}]_{\text{mito}}$ in egg cells is due to the difficulty of specifically targeting fluorescent Ca^{2+} indicators in the matrix of egg's mitochondria. The indicator Rhod 2 (which partitions into mitochondria of somatic cells and has been widely used to monitor $[\text{Ca}^{2+}]_{\text{mito}}$ [2,18,19,92], was used in the mouse egg [16]. This study claimed that $[\text{Ca}^{2+}]_{\text{mito}}$ oscillates together with $[\text{Ca}^{2+}]_{\text{cyto}}$ [16] but confocal imaging of Rhod 2 loaded mouse oocyte later suggested that this Ca^{2+} indicator does not partition into the mitochondria of these eggs [13].

Amusingly, the first measurement of $[\text{Ca}^{2+}]_{\text{mito}}$ in a living oocyte was that of ... rat heart mitochondria injected into a mature *Xenopus* oocyte [64]! Even though it is hard to infer what the situation is during fertilisation and whether a heart cell mitochondria behaves like an egg cell mitochondria, this study revealed that a mitochondrion in an oocyte can take up Ca^{2+} during a Ca^{2+} signal of similar size than the natural fertilisation signal and that Ca^{2+} influx into the mitochondria is dependent on both the mitochondrial electrical potential and the mitochondrial Ca^{2+} uniporter [64]. Yet, the Ca^{2+} buffering capacity of endogenous mitochondria in *Xenopus* oocytes was shown to participate in the coordination of periodic Ca^{2+} waves induced artificially in immature oocytes, strongly suggesting that endogenous mitochondria can indeed sequester Ca^{2+} in this species ([59,65]). However, the situation occurring during fertilisation of a mature *Xenopus* oocyte remains unknown.

In the middle of the 90s, a new generation of protein-based Ca^{2+} indicators that could be specifically targeted to the mitochondrial matrix appeared. They are based on the luminescent protein aequorin or on GFP [17,66]. While aequorin has been expressed in mitochondria of numerous somatic cell types [17,66], this strategy has surprisingly never been used in eggs. The GFP-based Ca^{2+} indicator called Pericam [67] can be expressed and imaged in mature mouse eggs and this strategy allowed us to observe oscillations of $[\text{Ca}^{2+}]_{\text{mito}}$ during sperm-triggered Ca^{2+} oscillations (R. Dumollard and J. Carroll, unpublished data). This observation suggests that cytosolic Ca^{2+} signals are readily transduced into mitochondrial Ca^{2+} signals in the mouse zygote. Application of this strategy to eggs of other species should confirm the universality of such phenomenon at fertilisation.

The tight regulation of $[\text{Ca}^{2+}]_{\text{mito}}$ during sperm-triggered Ca^{2+} signals raises the question of the roles of Ca^{2+} in the mitochondrial matrix and the impact of mitochondrial Ca^{2+} sequestering on egg activation. In fact, Ca^{2+} -dependent stimulation of mitochondrial respiration has been observed in eggs where

the $[Ca^{2+}]_{mito}$ could not be measured and these observations have been used as an indirect indication that mitochondria could sequester Ca^{2+} during sperm-triggered Ca^{2+} waves [13,15].

5. Impact of sperm-triggered Ca^{2+} waves on mitochondrial physiology

It has been known for a long time that Ca^{2+} in the mitochondria is a pivotal “multisite” activator of oxidative phosphorylation (Fig. 2); it stimulates several dehydrogenases of the Krebs cycle [69], the electron transport chain [68] and has a direct action on the F₀-F₁ ATP synthase [70]. It has thus been observed that an increase in $[Ca^{2+}]_{mito}$ can increase mitochondrial NADH (produced by the Krebs cycle) and mitochondrial ATP production [2,18,71]. Another indicator of oxidative phosphorylation is the degree of reduction (or the redox state) of the flavoproteins contained in enzymes of the Krebs cycle and in respiratory chain complexes [15,72]. Conveniently, the oxidised form of these flavoproteins (as well as NADH) is fluorescent and can be easily imaged in eggs [13,15,61,73,74].

The role of sperm-triggered Ca^{2+} waves in the stimulation of mitochondrial energy production at fertilisation was first suggested by the recording of an increase in oxygen consumption that peaked during sperm-triggered Ca^{2+} release in the sea urchin, starfish and ascidian egg [15,74,75]. However, in the sea urchin, this respiratory burst is mostly due to Ca^{2+} activation of cytosolic oxidases and therefore does not reflect mitochondrial respiration [76]. In the starfish, this respiratory burst is inhibited by the mitochondrial poison CN^- while, in the ascidian, the increase in oxygen consumption is associated with an increase in mitochondrial NADH suggesting that, in these species, mitochondrial respiration is indeed stimulated at fertilisation [15,74]. In the mouse egg, both NADH and oxidised flavoproteins oscillate in synchrony with the Ca^{2+} oscillations suggesting that Ca^{2+} in the mitochondria stimulates the reduction of NAD^+ and flavoproteins. After each Ca^{2+} transient, NADH and flavoproteins are then reoxidised slowly by the respiratory chain before the subsequent Ca^{2+} transient restarts a cycle of reduction thereby pacing ATP synthesis [13]. Cytosolic Ca^{2+} seems sufficient to stimulate mitochondrial respiration as eliciting an artificial Ca^{2+} signal induced in an unfertilised oocyte by uncaging IP₃ is able to stimulate oxygen consumption in the ascidian (Fig. 3A [15]) and the reduction of mitochondrial flavoproteins in the mouse (Fig. 3B [13]). However, the requirement for Ca^{2+} influx into the mitochondria could not be demonstrated due to the lack of effect of different blockers of the mitochondrial Ca^{2+} uniporter (R Dumollard, unpublished observation). Nevertheless, measurement of oscillations of $[Ca^{2+}]_{mito}$ using a mitochondrially targeted pericam supports the notion that, at fertilisation, an influx of Ca^{2+} into the mitochondria stimulates mitochondrial ATP production (R. Dumollard and J. Carroll, unpublished data).

Such measurements of CN^- -sensitive oxygen consumption coupled with imaging of mitochondrial NADH and flavoproteins in species different than sea urchin (which do not display the non-mitochondrial oxidative burst at fertilisation [74]) are needed to definitely establish the universal role of mitochondrial

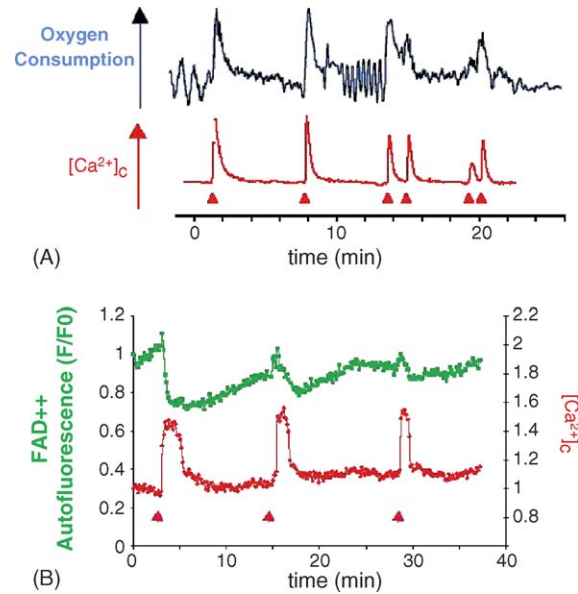


Fig. 3. Stimulation of mitochondrial respiration by cytosolic Ca^{2+} transients in eggs. (A) Ascidian (see [15]): variations of oxygen consumption in an ascidian egg (*Ascidia aspersa*) injected with caged Ins(1,4,5) P_3 (cIP₃). Simultaneous measurements of $[Ca^{2+}]_c$ (red trace) and oxygen consumption (blue trace) show that each time intracellular Ins(1,4,5) P_3 is photoreleased by a UV flash (red arrowhead), it induces a Ca^{2+} transient accompanied by a transient activation of oxygen consumption. (B) Mouse (see [13]): variations in $[Ca^{2+}]_c$ (measured with Rhod 2 AM, red trace) and FAD⁺⁺ autofluorescence (green trace) in a mature mouse egg injected with caged Ins(1,4,5) P_3 . A UV flash (red arrowhead) releases Ins(1,4,5) P_3 in the egg and triggers a Ca^{2+} transient accompanied by a transient decrease in FAD⁺⁺ autofluorescence.

Ca^{2+} in the metabolic activation of eggs at fertilisation. Alternatively, the possibility of monitoring $[ATP]_{cyto}$ and $[ATP]_{mito}$ in living fertilised eggs by recording the luminescence emitted by the natural ATP indicator luciferase [13,71,77] should confirm that the concerted effects of Ca^{2+} on mitochondria leads to an increase in ATP production necessary to support the increased energy demand associated with the activation of development.

Mitochondria are also a major source of ROS in the cell [78] and Ca^{2+} stimulation of mitochondrial oxidative phosphorylation leads to an increase in ROS production and probably a decrease in mitochondrial antioxidant defence [79,80]. In species other than sea urchin, such production of ROS by mitochondria could be significant and could have an impact on early development. So far direct measurement of ROS during fertilisation has not been performed but indirect evidence suggests that, in mammals, fertilisation is associated with an increase in oxidative stress since reduced glutathione (the major antioxidant of the cell) is decreased by 45% at the end of the sperm-triggered Ca^{2+} oscillations [81]. This indicates that oxidative events depleting reduced GSH occur after fertilisation. Importantly, the potentially damaging oxidative stress generated by mitochondria has to be minimised in order to prevent developmental demise, especially in the mammalian embryo which is very sensitive to oxidative stress [11,82,83]. The transient activation of mitochondrial metabolism sustained by repetitive Ca^{2+} waves observed in mammals and ascidians provides the major advantage of coupling the energy production to energy demand while minimally

stimulating the potentially detrimental oxidative metabolism of mitochondria [11,13,78,83].

The consequences of Ca^{2+} stimulated mitochondrial metabolism could first impact on the sperm-triggered Ca^{2+} waves themselves as intracellular Ca^{2+} release is known to be affected by ATP and oxidative stress [2,79,85,87,91].

6. Modulation of sperm-triggered Ca^{2+} waves by mitochondria

Sperm-triggered Ca^{2+} waves are generated mostly by IP₃-induced Ca^{2+} -release (IICR) (reviewed by Miyasaki, in this issue). IP₃-receptor (IP₃R) opening is regulated by Ca^{2+} , calmodulin, ATP as well as by the filling state of the ER, itself dependent on ATP hydrolysis [84,96]. Oxidative stress may also modulate IICR via a direct effect on the IP₃ receptor, on the Ca^{2+} pumps of the ER (SERCA) and the plasma membrane Ca^{2+} pumps (PMCA) [85–87] or even on IP₃ generation [88,89]. Mitochondria are able to regulate the levels of Ca^{2+} , ATP and ROS in the microdomain formed at the junction between ER and mitochondria present in oocytes (see the chapter “*Mitochondrial organisation, distribution and shapes in oocytes*”) and they may thereby regulate sperm-triggered Ca^{2+} waves (Fig. 2).

The impact of mitochondrial Ca^{2+} cycling on Ca^{2+} waves in eggs has been the most studied so far. An important feature of mitochondrial Ca^{2+} cycling is that Ca^{2+} uptake and efflux are distinct pathways (Fig. 2 [68]) the kinetics of the efflux being 10–100 times slower. In addition, Ca^{2+} influx is mediated by a Ca^{2+} uniporter of low affinity and is driven by the electrical potential while Ca^{2+} efflux is mainly mediated by an electroneutral $\text{Na}^+/\text{Ca}^{2+}$ exchange independent of the electrical potential [2,90]. Therefore, the net fluxes of Ca^{2+} into and out of mitochondria during a cytosolic Ca^{2+} transient are a rapid and large influx during the rising phase and the peak of the transient followed by a slow efflux during the decline of the Ca^{2+} transient [63,90] making mitochondrial Ca^{2+} transients generally longer lasting than the associated cytosolic transient [2,18,19].

The regulation of IP₃R opening by Ca^{2+} is dependent on the level of IP₃; under moderate IP₃ levels, a low [Ca^{2+}] promotes opening of the IP₃R whereas a high [Ca^{2+}] inhibits opening of the channel. On the other hand, under high IP₃ levels, the [Ca^{2+}] required to inhibit the IP₃R is much larger [84,91]. Thus, the apparent effect of mitochondrial Ca^{2+} cycling on intracellular Ca^{2+} waves generated by high IP₃ levels is a negative feedback on intracellular release observed as a desensitisation to IP₃, a slowing down of Ca^{2+} waves, of Ca^{2+} oscillations frequency or a decrease in peak amplitude [2,19,51,63,92]. Conversely, under low IP₃ levels, mitochondrial Ca^{2+} uptake can exert a positive feedback on intracellular release by delaying the inhibition of the IP₃R opening by Ca^{2+} [15,59,64].

In eggs, the negative impact of mitochondria on IICR is illustrated by the fact that Ca^{2+} puffs and initiation sites of repetitive Ca^{2+} waves induced by global and sustained increases of IP₃ and IP₃ analogs are observed in cytoplasmic regions of lowest mitochondrial density [51,57,95]. However, in ascidian eggs, global Ca^{2+} waves induced by uniformly elevated IP₃ still arise in the

mitochondria-poor animal pole region of the egg when the mitochondria are depolarised by FCCP (R. Dumollard, unpublished observation) suggesting that mitochondria decrease the sensitivity to IP₃ not because of their Ca^{2+} regulating function but because of their sole presence (i.e. by decreasing the ER density see [15,57,94]). Interestingly, in the mouse egg, the Ca^{2+} wave initiation sites induced by fertilisation or sperm extract injection are located in the vegetal cortex of the egg [93,95,100] which contains the lowest density of mitochondria ([13,54], Fig. 1). This suggests that a heterogeneous distribution of mitochondria may create a region of higher sensitivity to IP₃ in the vegetal cortex which becomes a preferential site for initiation of global Ca^{2+} waves [95]. However, it cannot yet be excluded that the vegetal Ca^{2+} wave pacemaker in mouse zygotes arises from preferential Ca^{2+} influx through the plasma membrane or from a specific spatial pattern of IP₃ production induced at fertilisation.

Inhibition of mitochondrial Ca^{2+} uptake at fertilisation by collapsing the mitochondrial electrical potential (with FCCP) also promotes mitochondrial ATP hydrolysis thus making it difficult to differentiate between effects on Ca^{2+} signals due to altered mitochondrial Ca^{2+} uptake or impaired mitochondrial ATP production. Indeed, even though mitochondria in sea urchin eggs accumulate enormous amounts of Ca^{2+} during the fertilisation Ca^{2+} wave, this fertilisation Ca^{2+} wave is not affected by the depolarisation of mitochondria [61]. This apparent lack of effect may be due to counteracting effects of inhibited mitochondrial Ca^{2+} uptake and of inhibited ATP production after mitochondrial depolarisation.

Fertilised ascidian eggs are characterised by two Ca^{2+} wave pacemakers (PM1 and PM2) triggering two series of repetitive Ca^{2+} waves associated, respectively, with the completion of meiosis I and II [15,94,95]. The first pacemaker (PM1) associated with the sperm entry site shows no specific relationship to mitochondrial distribution and is not affected by mitochondrial depolarisation [15]. On the contrary, pacemaker PM2, which is located in a cortical ER accumulation, juxtaposed to a large mitochondria-rich subcortical domain is rapidly and completely inhibited by mitochondrial depolarisation [15,94,95]. Our study showed that such inhibition of PM2 activity is not due to a global decrease in ATP but is due to the specific inhibition of mitochondrial Ca^{2+} uptake which probably maintains the IP₃R opened in response to the low IP₃ levels driving PM2 [15]. In contrast, in the mouse egg, mitochondria contribute to Ca^{2+} homeostasis and to the activity of the Ca^{2+} wave pacemaker by providing ATP but not by buffering Ca^{2+} [13].

ATP exerts a positive feedback on IICR as ATP^{4-} sensitises the IP₃R to IP₃ [91] and Mg-ATP^{2-} is consumed to refill the ER stores [96]. Accordingly it was found that mitochondrial ATP production participates in the activity of the ascidian pacemaker PM2 [15] and of the mouse pacemaker [13] but it is not known if this mechanism is at work in other species.

The generation of ROS (O_2^- then transformed into H_2O_2) by mitochondria could also modulate IICR in the egg (Fig. 2). The IP₃R is directly activated by sulfhydryl agents like thimerosal [21,85] or H_2O_2 [86] while ROS inhibit the SERCAs [85,87]. Moreover, IP₃ metabolism is slowed by H_2O_2 [87] and in *Xenopus* eggs H_2O_2 is able to stimulate Src and PLC-mediated IP₃

production [88,89]. In conclusion, if there is good evidence that oxidative stress can exert a positive feedback on IICR resulting in an increase in Ca^{2+} oscillations frequency in the mouse egg [20] and that mitochondria could be the source of such oxidative stress, it remains to be established that the oxidative metabolism of mitochondria is able to regulate sperm-triggered Ca^{2+} signals in eggs.

7. Perspectives

After the revelation more than 10 years ago that mitochondria in somatic cells could take up Ca^{2+} during physiological Ca^{2+} signals and modulate these signals, it has become clear that this phenomenon also operates in eggs during sperm-triggered Ca^{2+} waves. The discovery of an efficient mitochondrial Ca^{2+} uptake during sperm-triggered Ca^{2+} waves confers a new role in the activation of development for these Ca^{2+} signals which is to stimulate the energy production in the zygote. Even though such role of fertilisation Ca^{2+} waves has been studied in only few species so far, the fact that sperm-triggered Ca^{2+} signals stimulate mitochondrial respiration in starfish (echinoderm), ascidians (urochordate) and mice (vertebrate) suggest that this mechanism is conserved at least among deuterostomes. Monitoring sperm-triggered Ca^{2+} transients together with mitochondrial activity in other organisms (protostomes, cnidarians, ctenophores) will be necessary to confirm that activation of development by Ca^{2+} proceeds universally via stimulation of mitochondrial ATP production.

The vital role of the mitochondria in development is illustrated in the hypothesis that impaired mitochondrial metabolism may be responsible for the demise of embryonic development [11,16,29,97]. In aged mouse eggs, Ca^{2+} oscillations fail to increase ATP production [14] and induce apoptosis rather than development [22] suggesting that failure to activate mitochondrial metabolism may selectively abort embryonic development. Such a mechanism would ensure that only metabolically intact embryos are allowed to develop. The study of the relationships between sperm-triggered Ca^{2+} waves and mitochondrial metabolism should thus not only help to understand the processes of egg activation by Ca^{2+} but should also allow to predict for the first time how the metabolic state of the embryo regulates early development or determine developmental potential.

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