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Mitochondrial respiration and Ca^{2+} waves are linked during fertilization and meiosis completion

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SUMMARY

Fertilization increases both cytosolic Ca^{2+} concentration and oxygen consumption in the egg but the relationship between these two phenomena remains largely obscure. We have measured mitochondrial oxygen consumption and the mitochondrial NADH concentration on single ascidian eggs and found that they increase in phase with each series of meiotic Ca^{2+} waves emitted by two pacemakers (PM1 and PM2). Oxygen consumption also increases in response to Ins(1,4,5)P_3-induced Ca^{2+} transients. Using mitochondrial inhibitors we show that active mitochondria sequester cytosolic Ca^{2+} during sperm-triggered Ca^{2+} waves and that they are strictly necessary for triggering and sustaining the activity of the meiotic Ca^{2+} wave pacemaker PM2. Strikingly, the activity of the Ca^{2+} wave pacemaker PM2 can be restored or stimulated by flash photolysis of caged ATP. Taken together our observations provide the first evidence that, in addition to buffering cytosolic Ca^{2+}, the egg's mitochondria are stimulated by Ins(1,4,5)P_3-mediated Ca^{2+} signals. In turn, mitochondrial ATP production is required to sustain the activity of the meiotic Ca^{2+} wave pacemaker PM2.

Key words: Fertilization, Respiration, Ca^{2+} waves, Mitochondria, Endoplasmic reticulum, ATP, Ca^{2+} wave pacemakers, Ascidian

INTRODUCTION

Intracellular Ca^{2+} waves are triggered in somatic cells in response to neuromediators, hormones or growth factors (Berridge, 1997). In a wide variety of egg cells (from plant to human cells), fertilization triggers one to several Ca^{2+} waves. Repetitive Ins(1,4,5)P_3-mediated Ca^{2+} waves lasting over a period of 20 minutes to several hours (called 'meiotic Ca^{2+} waves') have been observed in ascidian, mammalian, starfish, molluscan, nemertean and annelid eggs at fertilization (reviewed by Sardet et al., 1998; Stricker, 1999; Dumollard et al., 2002). These Ca^{2+} signals are necessary for egg activation and progression through the meiotic cell cycles (Speksnijder et al., 1989; McDougall and Levasseur, 1998) (reviewed by Nixon et al., 2000). Repetitive Ca^{2+} signals are also thought to be important for early and post-implantation development in mammals (Jones, 1998; Ozil and Huneau, 2001).

Cellular Ca^{2+} homeostasis is the result of Ca^{2+} fluxes through the plasma membrane and the intracellular organelles. In every egg studied so far, an extensive and continuous ER network plays a major role in the sperm-triggered Ca^{2+} waves (reviewed by Sardet et al., 1998; Dumollard et al., 2002). The network hosts Ca^{2+} channels [Ins(1,4,5)P_3 receptors (IP_3R)], which mediate intracellular Ca^{2+} release, and Ca^{2+} pumps [sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs)]. Little is known about the role of mitochondria in the regulation of sperm-triggered Ca^{2+} signals in eggs. In sea urchins, mitochondria were suggested as a sink for Ca^{2+} released during fertilization (Eisen and Reynolds, 1985; Girard et al., 1991). A role for active mitochondria in the regulation of sperm triggered Ca^{2+} oscillations has been suggested in the mouse egg (Liu et al., 2001), but it cannot be inferred from this study what the interplay is between mitochondria and sperm-triggered Ca^{2+} oscillations.

In somatic cells, mitochondria can sequester and release Ca^{2+} during Ca^{2+} waves (Boitier et al., 1999; Rizzuto et al., 2000; Hajnoczky et al., 2000). Mitochondrial Ca^{2+} sequestering generally provides a negative feedback on Ins(1,4,5)P_3-induced Ca^{2+} release (IICR) (reviewed by Rizzuto et al., 2000; Duchen, 2000). Calcium is also a pivotal 'multisite' activator of oxidative phosphorylation in the mitochondria (Hansford, 1994). Calcium activates the dehydrogenases of the Krebs cycle (McCormack et al., 1990) and the electron transport chain (Gunter et al., 1994), and has a direct action on the F0-F1 ATP synthase (Territo et al., 2000). In somatic cells, mitochondrial Ca^{2+} uptake can increase the ratio of NADH/NAD^+ (i.e. NAD^+ is reduced to NADH) in the mitochondria (Pralong et al., 1994; Hajnoczky et al., 1995)

(reviewed by Duchen, 2000) as well as oxygen consumption (Duchen, 2000). Theoretically, mitochondrial ATP production can regulate IICR through ATP^{4-}, an allosteric regulator of IP_3R opening (Mak et al., 1999; Mak et al., 2001). Therefore active mitochondria may modulate Ca^{2+} signaling at two different levels either via preferential Ca^{2+} fluxes (reviewed by Duchen, 2000; Rizzuto et al., 2000; Hajnoczky et al., 2000) or via local ATP generation (Landolfi et al., 1998; Kennedy et al., 1999).

The first report of an increase in oxygen consumption after fertilization was a study on sea urchins (Warburg, 1908), which led to the common belief that fertilization is also accompanied by the metabolic activation of the egg. It is now recognized that respiration in sea urchin eggs and embryos depends solely on electron transport through the mitochondrial respiratory chain, except for a short period after fertilization when secreted ovoperoxidases consume O_2 to synthesize H_2O_2 (Perry and Epel, 1985; Heinecke and Shapiro, 1992; Yasumasu, 2000). In contrast to sea urchins and molluscs, this non-mitochondrial respiratory burst does not occur in asteroid, echiuroid or ascidian eggs (Schomer and Epel, 1998).

Given the role of Ca^{2+} waves in somatic cells (reviewed by Rizzuto et al., 2000; Duchen, 2000), sperm-triggered Ca^{2+} waves may mediate the metabolic activation of the fertilized egg, but this remains to be demonstrated. Fertilized ascidian eggs provide a particularly favorable model to investigate the relationships between meiotic Ca^{2+} waves and mitochondrial activity for several reasons.

(1) Fertilization does not involve a non-mitochondrial respiratory burst (Schomer and Epel, 1998).

(2) Thousands of rod-shaped mitochondria form a dense 7 μm subcortical layer lining the vegetal and equatorial regions of the egg (Roegiers et al., 1995; Roegiers et al., 1999).

(3) Fertilization triggers two series of very stereotyped Ca^{2+} waves which are generated by two distinct Ca^{2+} wave pacemakers (PM1 and PM2) (Sardet et al., 1998; Yoshida et al., 1998; Nixon et al., 2000; Dumollard and Sardet, 2001). Pacemakers PM1 and PM2 are located in the cortex of the egg (pacemaker PM2 is at the vegetal pole of the egg) and they are known to rely mostly if not only on a sperm-triggered Ins(1,4,5)P_3 production (McDougall and Sardet, 1995; Yoshida et al., 1998; Runft and Jaffe, 2000).

(4) A third artificial pacemaker (PM3) can be generated opposite to pacemaker PM2 (Dumollard and Sardet, 2001).

In this study, we have recorded two parameters of mitochondrial activity (oxygen consumption and mitochondrial NADH levels) during the period of activity of the Ca^{2+} wave pacemakers PM1, PM2 and PM3 of the ascidian egg. We have also examined the effects of mitochondrial inhibitors on the patterns of Ca^{2+} waves initiated by these three pacemakers. Our studies reveal that mitochondria are stimulated by cytosolic Ca^{2+} signals. In turn, the Ca^{2+} wave pacemaker PM2 strictly depends upon Ca^{2+} buffering and ATP production by mitochondria.

MATERIALS AND METHODS

Biological material

Specimens of the ascidian *Phallusia mammillata*, were collected either from Villefranche sur Mer or Sète (Mediterranean). The ascidians *Ciona intestinalis* and *Ascidiella aspersa* were purchased from the Marine Resource Center of the Marine Biological Laboratory in Woods Hole (MA, USA). The animals were kept in seawater at 16-22°C before use. Denuded oocytes were prepared, handled and fertilized as described previously (McDougall and Sardet, 1995; Roegiers et al., 1999).

Microinjection

Unfertilized eggs were introduced into a wedge and injected as described previously (McDougall and Sardet, 1995; Dumollard and Sardet, 2001). The cytosolic dyes Calcium Green dextran (10 kDa, CG) and Texas Red dextran (10 kDa, TR, Molecular Probes) as well as caged Ins(1,4,5)P_3 (cIP_3, Calbiochem), caged PtdIns(4,5)P_2 ($cgPIP_2$, Calbiochem) and NPE caged ATP (cATP, Calbiochem) were dissolved in injection buffer consisting of 180 mM KCl, 100 μM EGTA, 30 mM BES pH 7.1. Approximately 1% of the egg volume was injected to give final concentrations of 10-20 μM for CG and TR dextran; 5 μM for cIP_3; 35 μM for $cgPIP_2$; and 1 mM for cATP.

Photorelease of caged compounds and Ca^{2+} imaging

Injected oocytes were mounted in a perfusion chamber and imaged with a Leica Wild Leitz CLSM or TCS SP2 confocal microscope (McDougall and Sardet, 1995; Dumollard and Sardet, 2001). For UV photorelease of cIP_3, $cgPIP_2$ or cATP oocytes were co-injected with a mixture of CG and TR and the caged compound (Dumollard and Sardet, 2001). UV flashes were produced on the Leica confocal microscope using epifluorescence illumination (75 W mercury lamp). Some photorelease was also performed on an inverted Zeiss microscope (Axiovert 100 TV) essentially as described previously (Dumollard and Sardet, 2001).

NADH imaging

NADH fluoresces at 470 nm with peak absorption at 356 nm (Masters and Chance, 1993). A 470±20 nm bandpass filter (excitation: bandpass 360±10 nm, Chroma Opticals) was used to collect images of intracellular NADH concentration ([NADH]). The mitochondria-rich vegetal subcortex containing mostly mitochondria (Roegiers et al., 1995; Roegiers et al., 1999) (Fig. 5A,B) displayed a higher fluorescence (Fig. 2A). For each NADH image, the one pixel line running in the center of the entire mitochondria-rich domain (lines ab and a′b′ in Fig. 2A) was extracted by image analysis using Visilog software [details about the segmentation method can be obtained by contacting C. C. (cibert@ijm.jussieu.fr)]. To prevent motion artifacts, each extracted line of a NADH image (ab and a′b′ in Fig. 2A) was divided by the same line extracted in an essentially NADH-insensitive reference image [exc, bandpass 380± 10 nm; em, 470nm (Chroma Opticals)] acquired quasi simultaneously with the NADH image. These divided extracted lines (which are ratiometric measurements of $[NADH]_{mito}$) were stacked together to display the variations of $[NADH]_{mito}$ with time (running from left to right in Fig. 2A 'time image').

Measurement of oxygen fluxes with a self-referencing oxygen-sensitive vibrating microelectrode

Oxygen fluxes in a single egg were measured using the oxygen-sensitive self-referencing vibrating probe in the NVPF facility at the Marine Biological Laboratory in Woods Hole (MA, USA) (Land et al., 1999). The vibrating electrode was positioned within 5 μm of a denuded egg adhering to a coverslip in a petri dish (Fig. 1B). For fertilization experiments, activated sperm were added to the dish, while the egg was observed using time-lapse recording. The presence of Ca^{2+} waves was monitored by recording the wave of contraction that accompanies each Ca^{2+} wave (Roegiers et al., 1999). When indicated, FCCP was added to give a final concentration of 1 μM. In some experiments cIP_3 was photoreleased by UV illumination and $[Ca^{2+}]_c$ was monitored by recording the Calcium Green fluorescence using a Zeiss Atto Arc imaging system while simultaneously recording the oxygen fluxes with the oxygen probe.

Perfusion of mitochondrial inhibitors

The eggs were held under the microscope in a perfusion chamber and either FCCP (1 μM in sea water, Sigma), cyanide anion (CN^-; 2 mM in sea water, Sigma) or oligomycin (100 μM in sea water, Sigma) perfused. Washing of the inhibitors was carried out by performing several perfusions of sea water. The perfusions lasted less than 1 minute. During that period no recording could be made.

RESULTS

Global Ca^{2+} waves stimulate mitochondrial respiration in the egg and zygote of ascidians

Fertilization in ascidians elicits two stereotyped series of Ca^{2+} waves (Fig. 1A) initiated by two distinct Ca^{2+} wave pacemakers: PM1 and PM2 (Dumollard and Sardet, 2001) (examples of such repetitive Ca^{2+} waves can be seen on our web site at http://biodev.obs-vlfr.fr/biomarcell/ascidies/calcium.html). Calcium wave pacemaker PM1 starts at the point of sperm entry emitting first a sustained fertilization Ca^{2+} wave (lasting 3 to 4 minutes) followed by two to four smaller Ca^{2+} waves and the emission of the first polar body (pb1, Fig. 1A PM1). After a pause lasting 2 to 4 minutes, a second series of Ca^{2+} waves (for a period of 15-20 minutes) initiates in a vegetal protrusion called the contraction pole. The Ca^{2+} wave pacemaker PM2 produces these waves until the end of meiosis II (Fig. 1A PM2). Pacemaker PM2 is situated in a zone of cortical ER accumulation sandwiched between the plasma membrane and the 7 μm mitochondria-rich layer (see Fig. 5A). All meiotic Ca^{2+} waves traverse this layer of densely packed mitochondria.

To measure the oxygen fluxes around a single ascidian egg, we used an oxygen-sensitive, self-referencing microelectrode (Fig. 1B). This technique has been successfully used to measure local oxygen consumption in living cells and embryos (Land et al., 1999; Trimarchi et al., 2000). We found that the basal oxygen consumption in the vegetal pole region of the unfertilized ascidian egg was 38±10% higher than the oxygen consumption measured near the animal pole region of the same egg (data not shown, n=7). This is consistent with the presence of the bulk of mitochondria in a subcortical location in the vegetal hemisphere of the egg (see Fig. 5A).

Maximum oxygen consumption was measured by uncoupling mitochondria with the permeant protonophore FCCP that induces mitochondria to consume oxygen but also to hydrolyze ATP (see Fig. 3). Upon addition of 1 μM FCCP, the oxygen consumption of the egg increased more than six times in the example shown, reflecting the maximum rate of oxygen reduction by mitochondria (Fig. 1D). The graph shown in Fig. 1B represents a measurement made with the oxygen electrode positioned near the vegetal pole of the egg. Although there was some variability due to interspecimen differences or alternative positioning of the electrode, a general pattern of oxygen consumption was observed in the eight eggs examined (five *Phallusia* and three *Ciona*) (Fig. 1B). With the basal level of oxygen consumption before fertilization assigned a value of 100%, we observed that, on average, oxygen consumption rose during the activity of Ca^{2+} wave pacemaker PM1 to 167±18%, and then decreased during extrusion of polar body 1 to 140±15%. Oxygen consumption rose again to reach a maximum value during the period of Ca^{2+} wave pacemaker PM2 activity (173±14%). After the cessation of the meiotic Ca^{2+} waves, the oxygen consumption then stabilized to 126±11% (Fig. 1B).

To measure the impact of the rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) on oxygen consumption, we artificially raised $[Ca^{2+}]_c$ by photoreleasing injected cIP_3 with a global UV flash (Dumollard and Sardet, 2001). Increased oxygen consumption was associated with each Ca^{2+} transient caused by the photorelease of cIP_3 (Fig. 1C). There was no measurable delay between the onset of the Ca^{2+} transient and the onset of the oxygen consumption transient measured with the vibrating electrode. The decay of Ca^{2+} signal preceded the decrease in oxygen consumption (Fig. 1C).

Calcium can directly or indirectly activate several mitochondrial dehydrogenases of the Krebs cycle, thereby reducing the NAD^+/NADH pool (i.e. increasing the amount of reduced NADH and decreasing the oxidized NAD^+, Fig. 5D) (Duchen, 2000; Rizzuto et al., 2000). As NADH is endogenously fluorescent (Masters and Chance, 1993), we imaged NADH in a single ascidian egg (Fig. 2).

The vegetal subcortex of the egg contains mostly mitochondria (Fig. 5A,B) (Roegiers et al., 1995; Roegiers et al., 1999); we therefore chose to measure the variations of [NADH] in this mitochondria-rich domain to monitor the variations of $[NADH]_{mito}$. This domain is easily distinguished as a cortical crescent displaying higher NADH fluorescence (Fig. 2A, see Materials and Methods). As with oxygen consumption (Fig. 1B), variations of $[NADH]_{mito}$ measured in the middle of the mitochondria-rich region (corresponding to the vegetal contraction pole) (Roegiers et al., 1995) showed two main increases during the period of operation of the Ca^{2+} wave pacemakers PM1 and PM2 (Fig. 2B). Blocking the reduction of oxygen by the electron-transport chain with CN^- led to an increase in $[NADH]_{mito}$ (Fig. 2C). By contrast, perfusion of the uncoupler FCCP, caused a decrease in $[NADH]_{mito}$ in accordance with its presumed effect on the mitochondria (see Figs 3, 5). These observations imply that there is a strong positive correlation between meiotic Ca^{2+} waves and the activation of mitochondrial respiration.

Mitochondrial depolarization induces a Ca^{2+} leak in artificially activated or fertilized eggs

In order to examine the role of active mitochondria on the pattern, frequency and amplitude of Ca^{2+} waves in the ascidian egg, we used an array of mitochondrial inhibitors, which inhibit Ca^{2+} influx into mitochondria. Energized mitochondria are characterized by an electrochemical potential that is mainly powered by a pH gradient generated by the electron transport chain (Fig. 3E). This electrochemical gradient (around –150 mV) is the driving force for Ca^{2+} influx into the mitochondria via a Ca^{2+} uniporter (Gunter et al., 1994; Duchen, 2000). Calcium influx can be abolished by simply collapsing the pH gradient across the inner mitochondrial membrane (with CN^- or FCCP see Fig. 3G,H). Perfusion of unfertilized eggs with FCCP or CN^- for 10-20 minutes caused only a slight elevation of $[Ca^{2+}]_c$ (Fig. 3A, 0.03±0.02%, n=6, Fig. 3D). By contrast, when mitochondrial inhibitors were perfused after eggs have been activated by cIP_3 injection, they caused a long-lasting increase in the resting $[Ca^{2+}]_c$ (Fig. 3B). Upon washout of CN^-, $[Ca^{2+}]_c$ returned rapidly to its basal level (Fig. 3B).

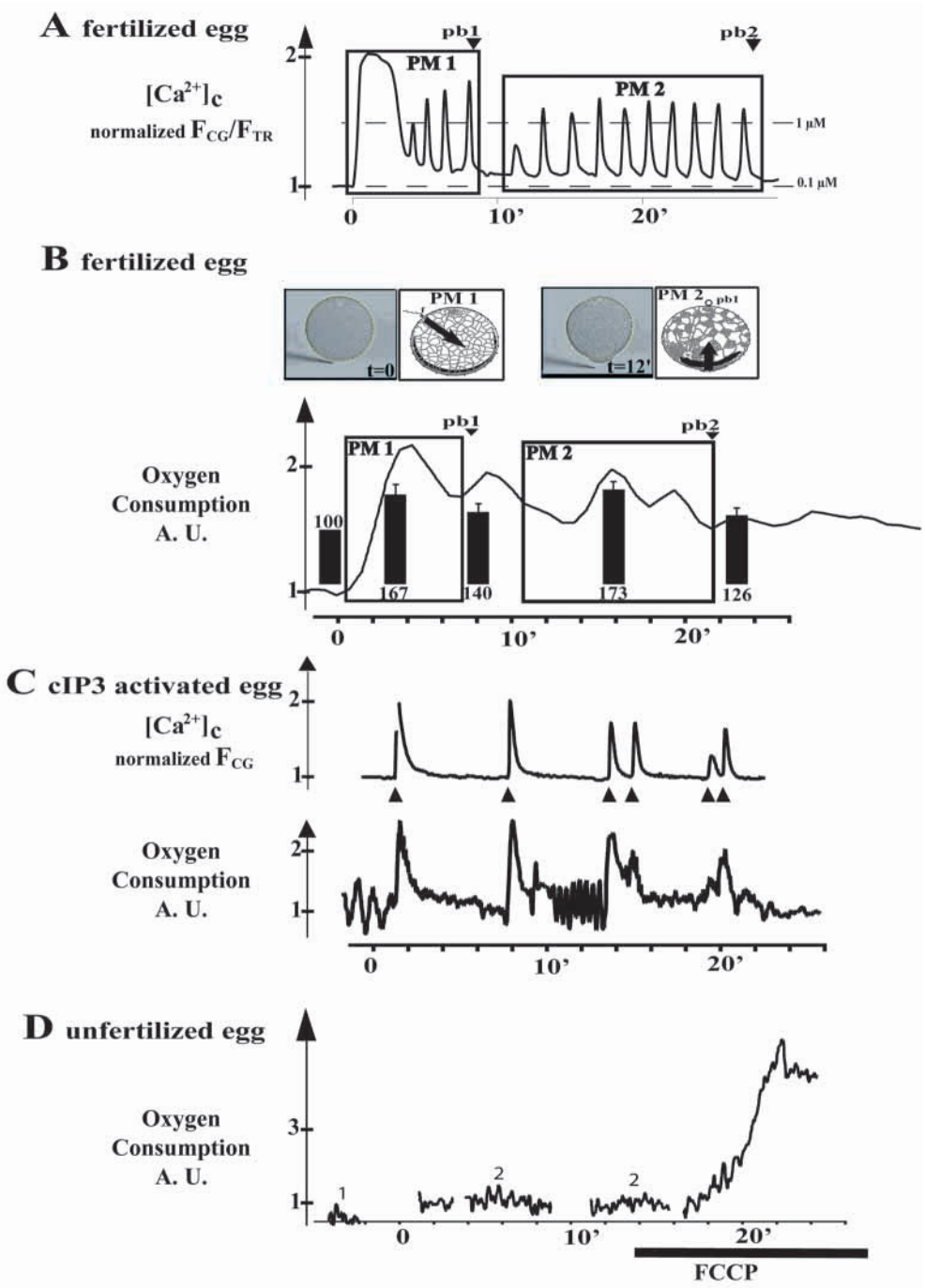

Fig. 1. (A) Sperm-triggered $[Ca^{2+}]_c$ oscillations during meiosis in an ascidian egg (*Phallusia*). The first series of Ca^{2+} oscillations is composed of the fertilization Ca^{2+} wave followed by four waves (PM1), which leads to the extrusion of the first polar body (pb1) about 5-7 minutes after sperm-egg fusion. After 2-4 minutes, the second series of Ca^{2+} oscillations (PM2) are triggered. These last 15-20 minutes, ending when the second polar body (pb2) is emitted. The values for $[Ca^{2+}]_c$ displayed on the right of the graph are from aequorin measurements (Speksnijder et al., 1989). (B) Variations of oxygen consumption during meiosis in ascidian eggs (*Phallusia*). Oxygen fluxes are measured on a single egg using the oxygen-sensitive self-referencing vibrating probe (upper images taken at t=0 and t=12 minutes). Sperm-egg fusion triggers a first increase in oxygen consumption, which peaks during the period of activity of Ca^{2+} wave pacemaker PM1 and lasts until the first polar body (pb1) is emitted. A second increase in oxygen consumption occurs during the period of activity of Ca^{2+} wave pacemaker PM2 and terminates when the second polar body is emitted (pb2) (n=7). The vertical bars in the graph represent average measurements of the oxygen consumption before fertilization (100%), after meiosis completion (126±15%) and during the activity of the pacemakers PM1 (167±18%) and PM2 (173±14%) (average from eight single eggs, five *Phallusia* and three *Ciona*). The transient increase in oxygen consumption observed between PM1 and PM2 in the example shown was not seen in other experiments. By contrast, two transient increases associated with the periods of pacemakers PM1 and PM2 activity were observed for every egg recorded. (C) Variations of oxygen consumption in an ascidian egg (*Ascidiella*) activated by caged Ins(1,4,5)P_3 (cIP$_3$) photolysis. Simultaneous measurements of $[Ca^{2+}]_c$ (using CG) and oxygen consumption (using the vibrating probe) show that each time intracellular Ins(1,4,5)P_3 is photoreleased by a UV flash (black arrowhead), it induces a Ca^{2+} transient accompanied by a transient activation of oxygen consumption (all 20 UV flashes applied to 6 different eggs generated similar responses in oxygen consumption). (D) Increase in oxygen consumption in an ascidian egg (*Ascidiella*) perfused with the mitochondrial uncoupler FCCP. The oxygen electrode is first positioned far from the egg (1) then brought close to the egg (2) to measure its basal oxygen consumption. A large increase in oxygen consumption is observed when FCCP is added to the dish to give a final concentration of 1 µM (bar under the graph).

The FCCP-induced increase of resting $[Ca^{2+}]_c$ was faster (0.25±0.05%, n=7, Fig. 3D) than the CN^--induced Ca^{2+} increase (0.10±0.04%, n=9). Interestingly, when Ca^{2+} transients were generated by cIP$_3$ photolysis, the continuous presence of CN^- or FCCP (for more than 10 minutes in the example shown in Fig. 3B) apparently did not affect the rate of the Ins(1,4,5)P_3-mediated Ca^{2+} rise or its decay (Fig. 3B is representative of 10 experiments).

The FCCP- or CN^--induced Ca^{2+} increase described above could be inhibited by preincubating the egg in seawater containing 100 µM oligomycin (which blocks the F0-F1 ATP synthase) for 20 minutes (Fig. 3C,D). This strongly suggests

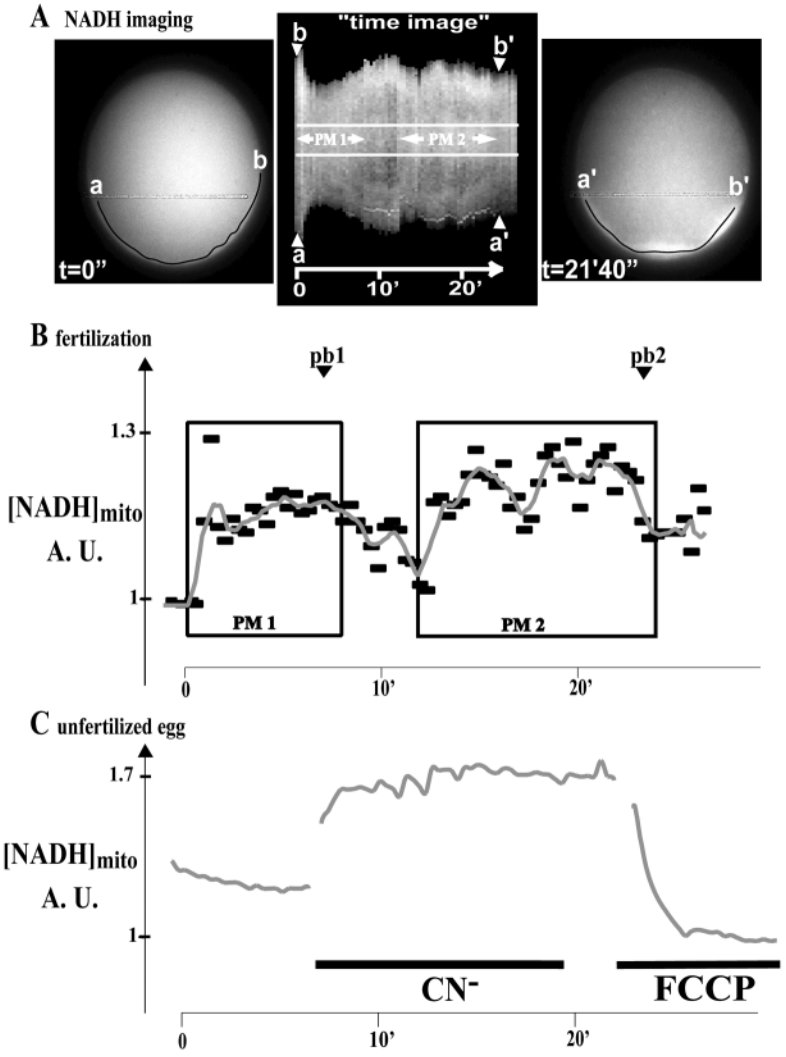

Fig. 2. Variations of the mitochondrial concentration of NADH ($[NADH]_{mito}$) in ascidian eggs (*Phallusia*). (A) Images of NADH of an egg before fertilization (left, t=0 minutes) and at the end of meiosis (right, t=21 minutes 40 seconds). The gray lines are artefacts caused by the CCD camera. In the 'time-image' (center), time (minutes) is measured on the x-axis and the y-axis corresponds to the extracted lines of the mitochondria-rich domain (a-b,a′-b′; see Materials and Methods). The varying length of each extracted line of mitochondrial NADH is due to acto-myosin driven cortical contractions traversing the egg during the activity of both Ca^{2+} wave pacemakers (Roegiers et al., 1999). The periods of activity of the two pacemakers are indicated by arrowheads; the scale goes from black for low [NADH] values to white for high [NADH] values. (B) Typical $[NADH]_{mito}$ variations observed after fertilization of an egg measured in the middle of the mitochondria-rich domain [between the two white horizontal lines in A (center)]. $[NADH]_{mito}$ increases during the period of activity of each Ca^{2+} wave pacemaker (PM1 and PM2) (n=8). (C) Variations of $[NADH]_{mito}$ after perfusion of an unfertilized egg with the mitochondrial inhibitors CN^- (2 mM) and FCCP (1 μm).

that the FCCP/CN^--induced Ca^{2+} increase was due to ATP depletion after the reversal of the mitochondrial ATP synthase when mitochondria were depolarized. The ATP depletion provoked by uncoupled mitochondria may well be responsible for an increased Ca^{2+} leak into the cytosol. Under these new net Ca^{2+} fluxes, the resting $[Ca^{2+}]_c$ equilibrates to a new, slightly higher level (Fig. 3B, Fig. 4C,E). The resting $[Ca^{2+}]_c$ did not increase further after 3 minutes of mitochondrial inhibitor perfusion, indicating that some Ca^{2+} extrusion from the cytosol could still operate (see Discussion).

Inhibition of mitochondrial activity has different effects on the three Ca^{2+} wave pacemakers

Fig. 4A,B,D illustrates the differential sensitivity of the two physiological pacemakers (PM1 and PM2) and of the artificial pacemaker PM3 (Dumollard and Sardet, 2001) to FCCP perfusion. Calcium wave pacemaker PM1 triggered by sperm entry was not affected by the presence of FCCP before or during fertilization (Fig. 4A, n=3), while the artificial pacemaker PM3 created by global photorelease of $cgPIP_2$ in the unfertilized egg was only slightly affected (Fig. 4D). We observed a gradual decrease of peak $[Ca^{2+}]_c$ and a gradual increase of inter-spike $[Ca^{2+}]_c$ about 4 minutes after the perfusion of FCCP (n=4, Fig. 4D). In contrast, the continuous presence of FCCP inhibited the pacemaker PM2 (n=3, Fig. 4A), which stopped emitting Ca^{2+} waves within seconds after the perfusion of FCCP (n=4, Fig. 4B). In addition, perfusing FCCP after the meiotic Ca^{2+} waves had ceased induced a transient elevation of $[Ca^{2+}]_c$ superimposed to the slow and sustained Ca^{2+} rise due to mitochondrial ATP hydrolysis (Fig. 4C, n=3; Fig. 4E, n=4). Importantly, this transient rise in $[Ca^{2+}]_c$ was localized to the vegetal hemisphere of the egg where the subcortical mitochondria-rich layer is located (data not shown). This FCCP-releasable Ca^{2+} pool was not present in the unfertilized egg (Fig. 3A) and it is different from the FCCP- or CN^--induced Ca^{2+} leak observed in eggs activated by Ins(1,4,5)P_3 or fertilized eggs. The most likely interpretation is that this Ca^{2+} pool resides in mitochondria that have sequestered and accumulated Ca^{2+} during the passage of the multiple Ca^{2+} waves triggered by PM1 and PM2.

CN^- had a similar effect to FCCP on the sperm-triggered pacemakers PM1 and PM2: pacemaker PM2 was inhibited, but PM1 was not perturbed (Fig. 4E, n=4; Fig. 4F, n=4). As with FCCP, PM2 activity recovered rapidly and completely upon washout of CN^- (Fig. 4E,F). The similar effects of the two different mitochondrial inhibitors on the different pacemakers demonstrate that active mitochondria are necessary for the maintenance of Ca^{2+} wave pacemaker PM2 but that they only play minor roles in sustaining the activities of pacemakers PM1 or PM3.

In order to examine the role played by the decreased level of ATP in the inhibition of pacemaker PM2, we used UV photolysis of NPE-caged ATP (He et al., 1998). Fertilized eggs were first exposed to CN^- and then flashed with UV light to increase $[ATP]_c$ globally. Increasing $[ATP]_c$ in fertilized eggs perfused with CN^- partially restored Ca^{2+} wave pacemaker PM2 activity (Fig. 4F). Global photorelease of ATP caused Ca^{2+} waves to initiate in the contraction pole where PM2 is

located (data not shown). However recovery of PM2 activity was only partial compared with the rapid and complete recovery observed after washout of CN^- (Fig. 4F). These observations suggest that mitochondrial ATP hydrolysis and the resultant decrease in $[ATP]_c$ accounts in part for the inhibition of the Ca^{2+} wave pacemaker PM2 by mitochondrial inhibitors.

The role of mitochondrial Ca^{2+} influx on the activity of the Ca^{2+} wave pacemaker PM2 was then investigated by perfusing CN^- on fertilized eggs in the presence of oligomycin. When incubated for 20 minutes in the presence of oligomycin, eggs were fertilized normally and the Ca^{2+} wave pacemakers PM1 and PM2 were not profoundly affected (Fig. 4G, n=3). CN^- perfusion during the period of Ca^{2+} wave pacemaker PM2 activity in these eggs still drastically inhibited PM2 (Fig. 4G) even though no ATP hydrolysis by mitochondria occurs under these conditions (see drawings in Fig. 3H,I). Therefore, the rapid inhibition of pacemaker PM2 is probably due to a rapid mitochondrial depolarization that blocks mitochondrial Ca^{2+} influx. Cytosolic Ca^{2+} buffering by the mitochondria may thus be crucial for Ca^{2+} wave pacemaker PM2 activity, particularly in the presence of oligomycin a situation during which no mitochondrial ATP synthesis can occur (see Discussion). Finally we photoreleased NPE caged ATP during PM2 activity, in order to characterize a potential regulation of PM2 activity by intracellular ATP during the normal operation of PM2 (i.e. without any mitochondrial inhibitors, Fig. 4H, n=4). The most striking effect of adding exogenous ATP during Ca^{2+} wave pacemaker PM2 activity was to decrease the period between successive waves elicited by pacemaker PM2 (bars in Fig. 4H show this decrease is of 21% in this experiment).

Together, these observations suggest that complex functional interactions between mitochondria and surrounding Ca^{2+}-release sites on the ER network occur during the operation of Ca^{2+} wave pacemaker PM2 and that they involve both local Ca^{2+} fluxes and mitochondrial ATP production.

DISCUSSION

This study provides the first experimental evidence that both meiotic Ca^{2+} waves and Ins(1,4,5)P_3-mediated Ca^{2+} signals can stimulate mitochondrial respiration in an egg. Therefore, meiotic Ca^{2+} waves not only control egg activation and the completion of the meiotic cell cycles (Spesknijder et al., 1989; McDougall and Sardet, 1995; Yoshida et al., 1998; Levasseur and McDougall, 1998), but they also stimulate the energetic metabolism of the zygote.

Our experiments with mitochondrial inhibitors and caged ATP also show that the stable vegetal Ca^{2+} wave pacemaker PM2, which operates in ascidian zygotes during completion of meiosis II, strictly depends on mitochondrial activity in contrast to pacemakers PM1 and PM3. Because Ca^{2+} wave pacemaker PM2 is located in a cortical domain of ER accumulation sandwiched between the plasma membrane and

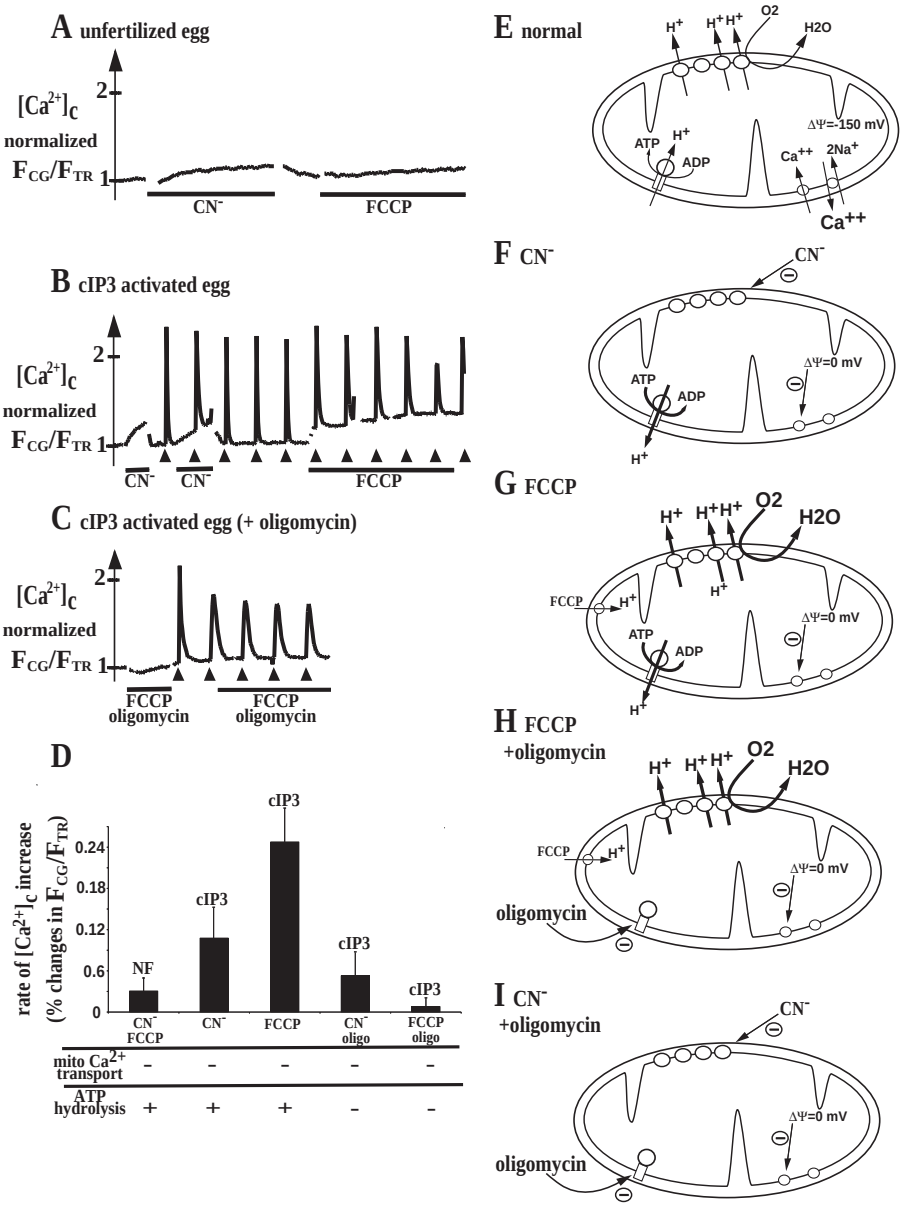

Fig. 3. Effect of mitochondrial inhibitors on Ca^{2+} levels and Ca^{2+} signals in ascidian eggs (*Phallusia*). Unfertilized eggs are injected with CG/TR only (A) or with CG/TR + 5 μM cIP_3 (B,C) are perfused with CN^- (C) and FCCP (B) with or without oligomycin. Ca^{2+} transients are induced every 3 minutes by a UV flash photoreleasing Ins(1,4,5)P_3 (black arrowheads). Rates of $[Ca^{2+}]_c$ increase induced by the three mitochondrial inhibitors on the egg are compared in the bar graph shown in D (n=17). (E-I) The actions of CN^- (F,I) and FCCP (G,H) on the mitochondria in the presence (H,I) or absence (F,G) of oligomycin, compared with the untreated egg (E).

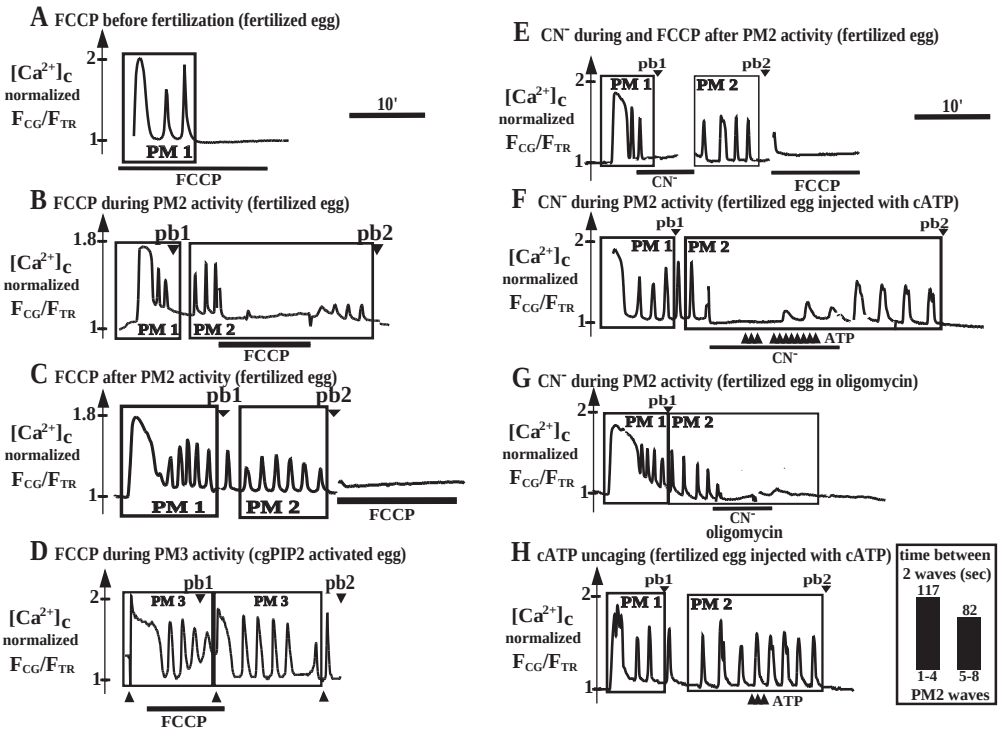

Fig. 4. Differential sensitivity of the three Ca^{2+} wave pacemakers of ascidian eggs (*Phallusia*) to mitochondrial inhibitors. (A--D) Effects of FCCP applied before fertilization (A, *n*=3) and during the period of activity of the Ca^{2+} wave pacemaker PM2 (B, *n*=4) inhibits PM2 activity. Perfusion of FCCP after extrusion of the second polar body (pb2) (C, *n*=3) produces a Ca^{2+} transient. Perfusion of FCCP during the period of activity of the Ca^{2+} wave pacemaker PM3 (D, *n*=4) affects pacemaker PM3 only slightly after 4 minutes. Perfusion of CN^- during the period of activity of PM1 (E) or PM2 (F,G) in eggs injected with CG/TR only (E, *n*=4) or with CG/TR and cATP (in F). Artificial production of ATP by UV flash photolysis of cATP (black arrowheads) restores partially PM2 activity (F, *n*=3). In eggs preincubated for 20 minutes in oligomycin before fertilization (G, *n*=3), Ca^{2+} wave pacemaker PM2 is still sensitive to CN^- perfusion. Artificial production of ATP by UV flash photolysis of cATP (black arrowheads) increases the frequency of the repetitive waves emitted by Ca^{2+} wave pacemaker PM2 (H, *n*=4). The periods for waves 1 to 4 (i.e. before the photorelease of ATP) and for waves 5 to 8 (i.e. after the photorelease of ATP) are indicated in the inset.

the subcortical mitochondria-rich domain (McDougall and Sardet, 1995; Roegiers et al., 1999; Dumollard and Sardet, 2001), we discuss our results in the light of possible functional relationships between ER and mitochondria.

Ca^{2+} waves activate mitochondrial respiration in ascidian eggs

Using two non-invasive recording techniques, we have been able to measure oxygen consumption and mitochondrial NADH levels of individual ascidian eggs. Basal oxygen consumption of the egg was highest in the vegetal pole region where the bulk of subcortical mitochondria are located (Roegiers et al., 1995; Roegiers et al., 1999; Dumollard and Sardet, 2001), suggesting that the oxygen consumption we measured was mainly due to mitochondrial respiration. The increases in both O_2 consumption and $[NADH]_{mito}$ observed at fertilization indicate that mitochondrial activity is stimulated by sperm entry.

Ca^{2+} appears to be the stimulus for increased mitochondrial respiration as both O_2 consumption and $[NADH]_{mito}$ increase during the activity of the Ca^{2+} wave pacemakers PM1 and PM2. Most remarkably, Ca^{2+} itself is sufficient to produce the increase in O_2 consumption as demonstrated by the transient burst of O_2 consumption in response to Ins(1,4,5)P_3. These data suggest that the repetitive Ca^{2+} waves emitted by the two pacemakers (PM1 and PM2) are responsible for the sperm-triggered activation of the energetic metabolism in the egg.

The activation of mitochondrial metabolism by the sperm-triggered Ca^{2+} waves suggests that, in these eggs, mitochondria take up cytosolic Ca^{2+} during the passage of a Ca^{2+} wave. The appearance of a FCCP-releasable Ca^{2+} pool after the activity of the pacemakers PM1 and PM2 further supports such hypothesis. Indeed after meiotic Ca^{2+} oscillations have occurred, FCCP perfusion induced a Ca^{2+} transient in the vegetal region of the egg (superimposed on the slow and sustained increase in $[Ca^{2+}]_c$ induced by FCCP or CN^- perfusion in artificially activated or fertilized eggs) that most probably reflected release of Ca^{2+} from the mitochondria upon mitochondrial depolarization.

Although mitochondria were suggested to be a sink for the Ca^{2+} released during the fertilization wave in sea urchin eggs (Eisen and Reynolds, 1985; Girard et al., 1991), no link to mitochondrial respiration could be established because of the concomitant non-mitochondrial respiratory burst occurring in these eggs at fertilization (Schomer and Epel, 1998; Yasumasu, 2000). In a very recent study, mitochondrial Ca^{2+} has been suggested to play a role in the control of mitochondrial respiration in sea urchin eggs (Fujiwara et al., 2001). However this study predicts that a high $[Ca^{2+}]_{mito}$ in the unfertilized sea urchin egg would inhibit mitochondrial respiration (Fujiwara et al., 2001), which is in complete contradiction with the previous studies on somatic cells, on sea urchins (Eisen and Reynolds, 1985; Girard et al., 1991) and with our findings in ascidian eggs.

Inhibition of the Ca^{2+} wave pacemaker PM2 by mitochondrial inhibitors is not due to a global depletion in ATP levels

The presence in ascidian eggs of three Ca^{2+} wave pacemakers with distinct locations and Ca^{2+} wave patterns provides an ideal model system with which to study the influence of mitochondrial activity on the regulation of intracellular Ca^{2+} waves. Perfusions

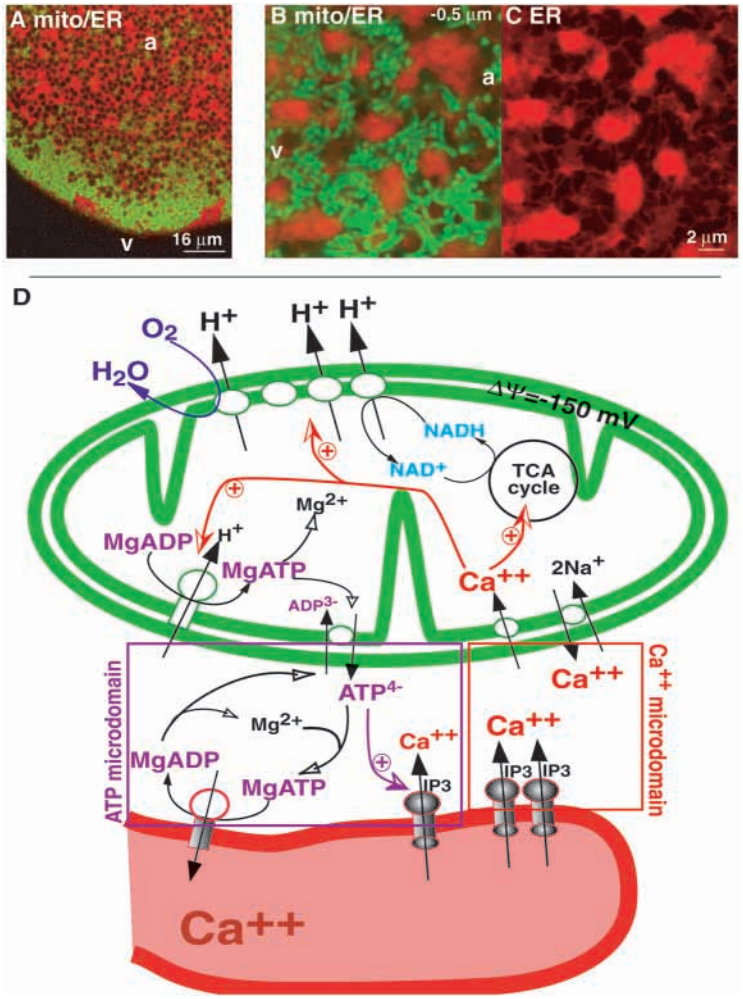

Fig. 5. Interactions between mitochondria and ER in the ascidian egg and in the Ca^{2+} and ATP microdomain. (A-C) Imaging of the ER network (red) and of mitochondria (green) in the vegetal contraction pole (v) of a fertilized *Phallusia* egg (A) shows the cortical ER-rich domain closely apposed to the subcortical mitochondria-rich domain. At higher resolution (B), rod-shaped mitochondria are observed densely packed in the vegetal subcortex (0.5 µm under the surface of the egg). Mitochondria are in close proximity to ER-rich domains and tubes of ER (C; reveals ER tubes between ER-rich domains shown in B). a, animal side. (D) Probable Ca^{2+} fluxes in the Ca^{2+} microdomain forming at the interface between a mitochondria (green) and an ER tubule (red) with clustered IP_3Rs. In the same space an ATP microdomain is formed between mitochondria (producing and exporting ATP) and Mg-ATP-consuming pumps (SERCAs) and ATP^{4-}-using channels (IP_3Rs). Calcium released in the cytosol can be sequestered by the mitochondria where it stimulates oxidative phosphorylation. ATP^{4-} is exported from the mitochondria into the cytosol where it stimulates Ca^{2+} release by sensitizing the IP_3Rs to Ca^{2+}. Finally, Mg-ATP generated by mitochondria can energize Ca^{2+} pumping back into the ER lumen and replenish Ca^{2+} stores.

of mitochondrial inhibitors during the period of activity of the different pacemakers revealed that, whereas Ca^{2+} wave pacemaker PM2 was immediately and completely blocked upon FCCP or CN^- perfusion, the pacemakers PM1 and PM3 were only slightly altered after lengthy perfusions of these mitochondrial inhibitors. Several observations suggest that the rapid inhibition of the Ca^{2+} wave pacemaker PM2 is not caused by emptying Ca^{2+} stores due to a global depletion in ATP levels. First, the activated egg could still respond to Ins(1,4,5)P_3 after more than 20 minutes of FCCP perfusion and the fertilized egg could respond to Ins(1,4,5)P_3 during PM2 inhibition by FCCP or CN^- (R. D., unpublished). Second, the resting $[Ca^{2+}]_c$ remained relatively low for long periods of time during metabolic inhibition of the pacemaker PM2, implying that ATP-dependent Ca^{2+} extrusion from the cytosol was still operating, with a part of it probably refilling the ER Ca^{2+} stores. Therefore, mechanisms other than global ATP depletion must account for the inhibition of Ca^{2+} wave pacemaker PM2 by mitochondrial inhibitors.

Mitochondrial modulation of the Ca^{2+} wave pacemaker PM2

In most eggs studied so far, Ins(1,4,5)P_3-induced Ca^{2+} release from the ER Ca^{2+} stores supports the repetitive Ca^{2+} waves observed at fertilization. Specifically in ascidians, it is known that injecting heparin (McDougall and Sardet, 1995) or the function blocking antibody specific to the IP_3R1 (18A10) (Yoshida et al., 1998) both inhibit the pacemaker PM2. Moreover, blocking specifically PLCγ activity inhibits both pacemakers (Runft and Jaffe, 2000) further illustrating that the pacemakers PM1 and PM2 rely mostly if not only on a sperm-triggered Ins(1,4,5)P_3 production. Given the central role of Ins(1,4,5)P_3 mediated Ca^{2+} release in PM2 activity, IP_3Rs may be a key target for the regulation by mitochondria. There are at least two mechanisms by which mitochondria can regulate the opening of the IP_3R channels: first, by locally buffering $[Ca^{2+}]_c$; second, by controlling the local concentration of ATP (as $MgATP^{2-}$ and ATP^{4-} ions).

In recent years it has been established that mitochondria sense the high $[Ca^{2+}]_c$ in the vicinity of the IP_3Rs and take up significant amount of Ca^{2+} during the passage of Ca^{2+} waves (Hajnoczky et al., 2000; Duchen, 2000, Rizzuto et al., 2000). In such 'Ca^{2+} microdomains' created between clusters of IP_3Rs and juxtaposed mitochondria (Fig. 5D), the local Ca^{2+} buffering provided by mitochondria can promote IP_3R opening or closure depending on a bell shape sensitivity to Ca^{2+} at a given $[Ins(1,4,5)P_3]_c$ (i.e. Ca^{2+} above a low threshold level promotes opening, while higher Ca^{2+} levels inhibit IP_3R opening). At low Ins(1,4,5)P_3 levels, moderate Ca^{2+} levels inhibit IP_3R opening, while under a higher concentration of Ins(1,4,5)P_3, higher levels of Ca^{2+} are required to inhibit IP_3R opening (Bootman and Lipp, 1999; Mak et al., 1999; Mak et al., 2001).

Our data suggest that inhibiting mitochondrial Ca^{2+} accumulation during PM2 activity using CN^-, while blocking mitochondrial ATP hydrolysis with oligomycin rapidly stops the ability of the pacemaker PM2 to generate Ca^{2+} waves. This finding is consistent with the hypothesis that at the low Ins(1,4,5)P_3 levels driving the pacemaker PM2 (Dumollard and Sardet, 2001; Dumollard et al., 2002), Ca^{2+} buffering by mitochondria is necessary to keep the local $[Ca^{2+}]_c$ in the activating part of the bell shape curve. The reason why PM1 is insensitive to inhibition of

mitochondrial Ca^{2+} accumulation is that it is driven by a larger increase in Ins(1,4,5)P_3 levels induced at the activation wave (Dumollard and Sardet, 2001; Dumollard et al., 2002). Under these conditions of high Ins(1,4,5)P_3 levels, the $[Ca^{2+}]_c$ must reach higher levels to inhibit IP_3Rs opening and Ca^{2+} buffering by mitochondria would not be so crucial.

A role for local control of ATP is indicated by the finding that photoreleasing ATP^{4-} in eggs in which PM2 was inhibited by CN^- perfusion caused a reinitiation of Ca^{2+} waves from the contraction pole. Mitochondrial ATP production in an 'ATP microdomain' (as shown in Fig. 5D) can modulate intracellular Ca^{2+} release (Yang and Steele, 2000). ATP^{4-} is an allosteric regulator of IP_3R1 opening (Mak et al., 1999; Mak et al., 2001) the predominant IP_3R in the egg cell (Fissore et al., 1999; Brind et al., 2001) and also the most sensitive to ATP (Miyakawa et al., 1999; Maes et al., 2000). Electrophysiological studies have revealed that ATP^{4-} (but not the $MgATP^{2-}$ complex) sensitizes the IP_3R to activation by Ca^{2+} (Mak et al., 1999; Mak et al., 2001). Moreover when $[Ins(1,4,5)P_3]_c$ is low, ATP^{4-} can also suppress Ca^{2+}-dependent inhibition of the channel opening (Mak et al., 2001). During mitochondrial inhibition of the pacemaker PM2, some of the photoreleased ATP^{4-} will directly bind to the IP_3Rs, thereby suppressing Ca^{2+}-dependent inhibition of the channels and rendering the vegetal contraction pole region excitable for the initiation of global Ca^{2+} waves. In addition, the remaining ATP^{4-} ions will bind Mg^{2+} ions and form $MgATP^{2-}$ complexes that can be used by SERCAs to refill the ER Ca^{2+} stores (Fig. 5D). Such a model does explain why, while PM2 is completely blocked by mitochondrial inhibition, the egg can still respond to the rather high Ins(1,4,5)P_3 increases achieved by uncaging cIP_3 (between 100 and 500 nM after each UV flash) (Dumollard and Sardet, 2001) and PM1 (which functions under high Ins(1,4,5)P_3 levels) is not altered by mitochondrial inhibition. This model can also account for the increase in frequency of Ca^{2+} oscillations generated by PM2 after the photorelease of ATP^{4-}. As the threshold $[Ca^{2+}]_c$ for triggering a wave can be lowered by increased ATP^{4-} (Mak et al., 1999; Mak et al., 2001) this threshold can be reached more quickly after a wave.

Taken together, our observations and the above considerations indicate that functional interactions between ER and mitochondria occur during sperm-triggered Ca^{2+} waves via two mechanisms: local Ca^{2+} fluxes between the two organelles and mitochondrial production of ATP. While local Ca^{2+} buffering in the 'Ca^{2+} microdomain' may suppress the Ca^{2+} inhibition of the IP_3R, local ATP production in the 'ATP microdomain' would sensitize and potentiate IICR (Fig. 5D). Even though this work raises the possibility that both modes of communication between ER and mitochondria are at work during the generation of sperm-triggered Ca^{2+} waves, only precise measurement of $[ATP^{4-}]_c$, $[MgATP^{2-}]_c$, $[Ca^{2+}]_c$ in the 'Ca^{2+} and ATP microdomain' in the living zygote will allow us to define the complex roles mitochondria play in the regulation of the meiotic Ca^{2+} wave pacemakers.

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