Zygote **9** (November), pp 347–352. © 2001 Cambridge University Press Printed in the United Kingdom

Confocal microscopy analysis of the activity of mitochondria contained within the 'mitochondrial cloud' during oogenesis in *Xenopus laevis*

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Date submitted: 17.4.01. Date accepted: 15.5.01

Summary

We have used ratiometric confocal microscopy and three fluorescence techniques to study the distribution and activity of mitochondria in frog oocytes during the early stages of oogenesis. Mitochondria in frog oocytes during oogenesis were characterised by a high ratio in the 'mitochondrial cloud' and perinuclear region and a low ratio in mitochondria freely dispersed within the cytoplasm. We tested whether the high ratio visualised by the three techniques represented mitochondrial membrane potential by perturbing the mitochondrial membrane potential. Carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone (FCCP) caused the immediate destruction of the membrane potential, and consequent loss of fluorescence from the membrane-potential-sensitive confocal channel. In contrast, nigericin caused an increase in membrane potential represented by a steady increase in fluorescence ratio. These data demonstrate that mitochondrial activity can be measured during oogenesis in frog oocytes, and suggest that the mitochondrial cloud and perinuclear regions are characterised by highly active mitochondria.

Keywords: ATP production, Mitochondrial membrane potential, Oocyte polarity, Oogenesis

Introduction

One of the early features of frog oogenesis is the formation of a clearly visible structure next to the germinal vesicle (Raven, 1961). This body, known as the Balbiani body or 'mitochondrial cloud' is formed during stage I of oogensis (stages according to Dumont, 1972). The mitochondrial cloud is stable during stage I, then fragments during stage II into zones which demarcate the future vegetative pole (Heasman *et al.*, 1984). The mitochondrial cloud appears to organise the localisation of distinct RNA species such as Xcat2, Xwnt11, Xlsirts (Kloc *et al.*, 1996) and Xotxl (Pannese *et al.*, 2000) in the future vegetal pole of the oocyte through the messenger transport organiser (METRO). METRO is a microtubule- and microfilament-independent process that causes the localisation of RNAs (Kloc & Etkin, 1995; Kloc *et al.*, 1996) to this region during the early stages of oogenesis. In contrast, the late transport system is microtubule- and microfilament-dependent (Yisraeli *et al.*, 1990) and causes the localisation of Vg1 RNA, implicated in the organisation of embryo symmetry (Thomsen & Melton, 1993). The mitochondrial cloud contains the germ plasma (Heasman *et al.*, 1984) and in particular germinal granules where Xcat2 is located and a fibrillar network where Xlsirts and Xwnt11 are found (Larabell, 1993).

The mitochondrial cloud is an aggregation highly enriched with mitochondria (Heasman *et al.*, 1984) that derives from the coalescence of several mitochondrial aggregates during early stage I of oogenesis (Wylie *et al.*, 1985) through a poorly defined process. Apart from mitochondria, the body is known to contain both cytokeratin (Wylie *et al.*, 1985) and spectrin proteins, as well as a γ-tubulin-containing body (Kloc & Etkin, 1998; R. Carotenuto, personal communication).

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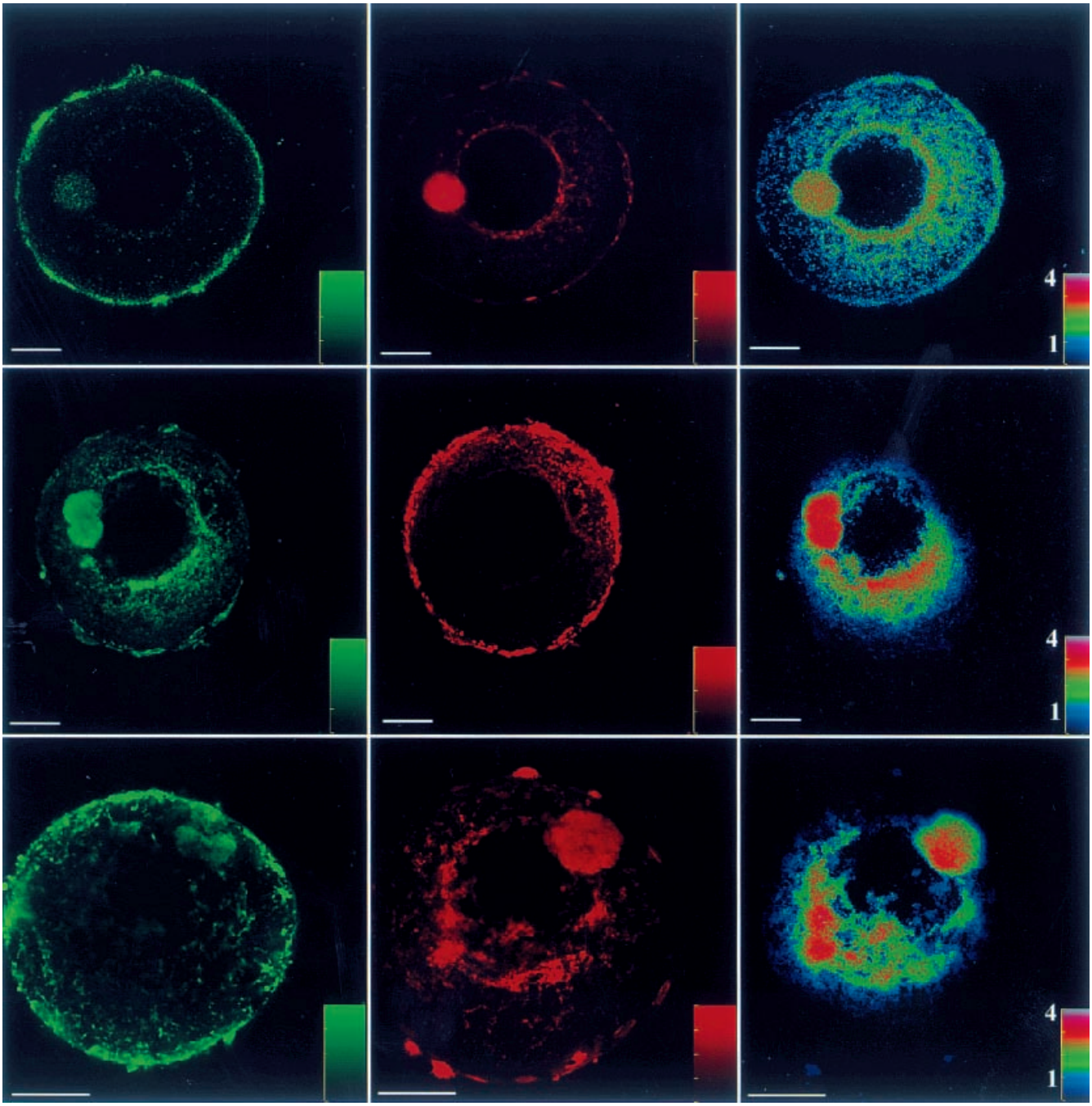

Figure 1 Analysis of mitochondrial activity in living frog stage II oocytes. Images show the green and red fluorescence outputs of the raw data in original colours and the rationed analysis of the experiment in false colour look-up tables. Top images are a representative example of an experiment where JC-1 was used as the fluorescence dye (technique I). Centre images are experiments where rhodamine-123 was used in conjunction with Mitotracker Red (technique II). Bottom images represent experiments where $DiOC_2(3)$ was used together with Mitotracker Green (technique III). Scale bars represent 50 μm.

Furthermore, lipid droplets grow in number in the cloud during oogensis, and data indicate that also *fatvg*, an mRNA encoding for protein probably involved in the utilisation of yolk platelets (Chan *et al.*, 1999), is located in the mitochondrial cloud, suggesting an energy-utilization activity during early oogensis.

In this study we used ratiometric confocal microscopy to examine the activity of mitochondria in the frog oocyte cytoplasm during early oogenesis. We compare the results obtained using a variety of techniques. Our data demonstrate that mitochondria located within the mitochondrial cloud are highly

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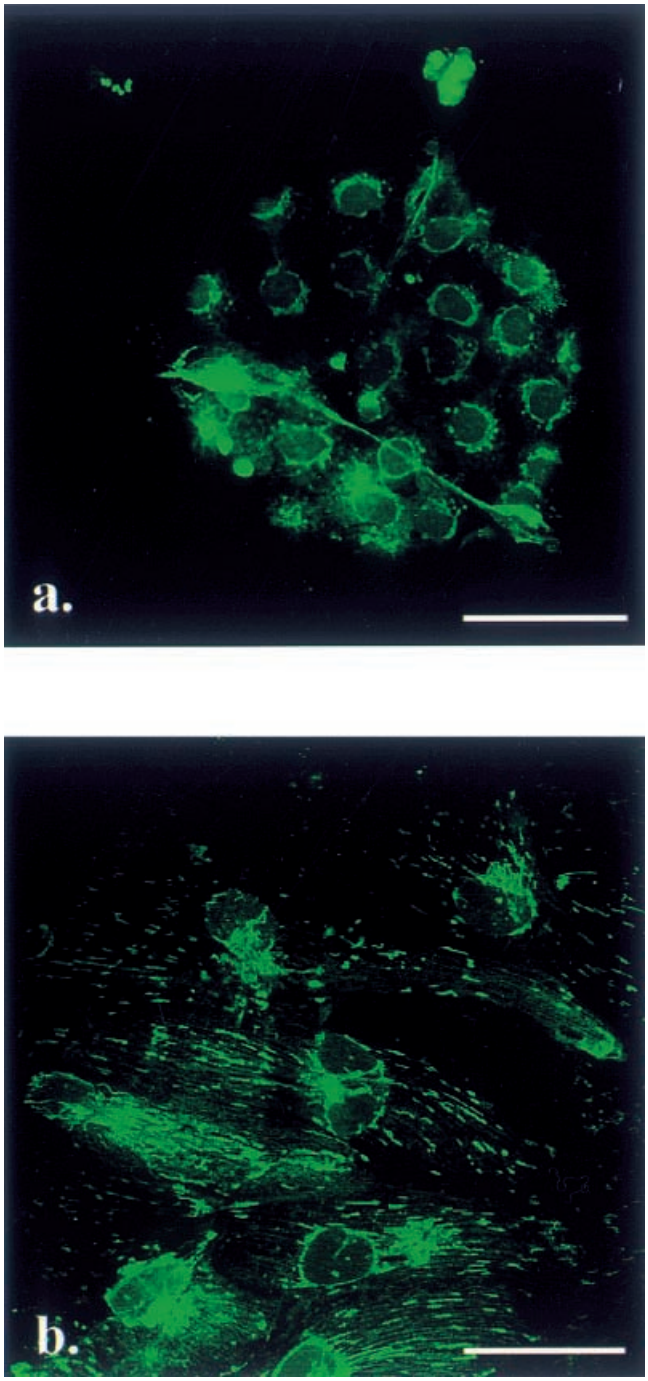

Figure 2 Mitochondria in follicle cells surrounding frog oocytes. Rhodamine-123 was used to label oocytes and confocal scans taken towards the periphery of the oocyte. Green fluorescence represents rhodamine-123. (*a*) Images taken at ×40 power magnification reveal follicle cells at the periphery of the oocyte. The image is representative of 12 experiments. Scale bar represents 50 µm. (*b*) High-power scan (×60 magnification) reveals filaments between follicle cells. Images are representative of 12 experiments. Scale bar represents 50 µm.

active compared with mitochondria dispersed within the cytoplasm. These data suggest that the mitochondrial cloud is a region of high activity in frog oogenesis.

Materials and methods

Oocytes

Adult females of the frog *Xenopus laevis* were obtained from 'Rettili' (Varese, Italy). Ovarian tissue was obtained by dissection after anaesthesia using MS222. Tissue was treated with 10 µg/ml proteinase K, 20 IU/ml hyaluronidase and 2 mg/ml collagenase in Barth's salts (88 mM NaCl, 1 mM KCl, 24 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 10 mM Hepes, pH 7.4) for 30 min, followed by manual selection of stage I and II oocytes (80–450 µm diameter) using a mouth pipette. Oocytes were washed twice in enzyme-free Barth's solution before use, and occasionally stored overnight at 4 °C.

Ratiometric confocal microscopy

Mitochondrial activity was measured using an Olympus Fluoview confocal microscope based on an Olympus IX70 inverted microscope equipped with an argon/krypton laser. Laser lines of 488 nm were used to excite rhodamine-123 and 568 nm for Mitotracker Red. Crosstalk between the green and red channels was negligible using this system. Images were divided pixel by pixel to obtain a ratio of the rhodamine-123 fluorescence to Mitotracker fluorescence. Images were further processed using the Fluoview software, or Adobe Photoshop.

Results

Mitochondrial activity is localised to the mitochondrial cloud

We tested the localisation and activity of mitochondria in frog stage I oocytes by using a variety of ratiometric confocal techniques. These included the dual emission probe, JC-1 (technique I), a dual-probe system consisting of rhodamine-123 and Mitotracker Red (technique II) and an alternative system consisting of $DiOC_2(3)$ and Mitotracker Green (technique III). The three systems were all characterised by high-intensity localisation of mitochondria to the mitochondrial cloud and periphery of the germinal vesicle, and low-intensity diffuse fluorescence in the remainder of the cytoplasm (Fig. 1). Where analysed, mitochondria were also observed in the follicle cells surrounding the oocyte (Fig. 2*a*). High-resolution imaging revealed these cells to be connected by mitochondria-containing protru-

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sions, suggesting that the oocyte is surrounded by a strongly interconnected cellular system (Fig. 2*b*).

Owing to the need to standardise results obtained with the three techniques, we normalised the preparation conditions. In technique I, oocytes were incubated for 30 min in Barth's salts into which JC-1 was dissolved to 0.5 μM. For technique II, oocytes were pre-incubated for 1 h in 10 μM Mitotracker Red dissolved into Barth's salts, followed by 30 min incubation in 200 μM rhodamine-123. The protocol for technique III involved preincubation of oocytes in Barth's salts containing 10 μM Mitotracker Green for 1 h followed by 30 min incubation in Barth's salts containing 1 μM $DiOC_2(3)$. Technique III was further characterised by maintaining oocytes in 1 μM $DiOC_2(3)$ during the experiment.

Ratiometric confocal microscopy not only enables the localisation of mitochondria but also permits the visualisation of regions of high mitochondrial activity. We examined whether frog oocytes were characterised by localised heterogeneity in mitochondrial activity by dividing images accumulated with the potential-sensitive mitochondria channel pixel-by-pixel by images from the same experiment representing mitochondrial distribution. In all the techniques, mitochondrial activity was observed to be higher in the mitochondrial cloud and germinal vesicle region than in mitochondria dispersed throughout the cytoplasm (Fig. 1, Table 1). Mitochondrial activity was 3- to 4-fold greater in these regions (Table 1). These data suggest that mitochondria localised within the mitochondrial cloud and germinal vesicle region are characterised by a higher state of activity than non-localised mitochondria.

Table 1 Ratiometric analysis of mitochondrial activity within localised mitochondria

	Non-localised mitochondria	Mitochondrial cloud and germinal vesicle region
Technique I (n = 11)	1.0 ± 0.2	3.8 ± 0.1
Technique II (n = 7)	0.8 ± 0.1	4.2 ± 0.2
Technique III (n = 8)	0.9 ± 0.2	3.2 ± 0.4

Data are represented as mean ± standard deviation.

Different ratiometric techniques are characterised by diverse responses to perturbations in mitochondrial activity

We tested whether the initial ratio measured by the three techniques could be defined as mitochondrial activity by using drugs to perturb the mitochondrial membrane potential. To destroy mitochondrial membrane potential, we added the mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone (FCCP) and observed the effects of this drug on the ratio. We tested the effects of FCCP on frog oocytes after loading with each of the three ratiometric techniques. In the presence of JC-1 (technique I), green fluorescence was rapidly lost after the addition of FCCP (Fig. 3*a*). From our measurements, we calculated the $t_{\frac{1}{2}}$ of the loss of the fluorescence ratio to be 80 ± 23 s (mean ± SD; n = 6; Fig. 3*a*). Ratiometric technique II, involving the combination of rhodamine-123 and Mitotracker Red, was also characterised by the loss of green fluorescence when FCCP was added to the chamber. In these experiments, the $t_{\frac{1}{2}}$ of the loss of the fluorescence ratio was calculated to be 62 ± 16 s (mean ± SD, n = 14; Fig. 3*b*). The loss of the fluorescence due to the addition of FCCP in oocytes stained with technique III was slower than that of the previous techniques. The $t_{\frac{1}{2}}$ was calculated to be 165 ± 42 s (mean ± SD, n = 12; Fig. 3*c*).

When the ionophore nigericin, which artificially increases mitochondrial membrane potential through K^+/H^+ exchange across the inner mitochondrial membrane (Johnson *et al.*, 1981), was added to frog oocytes loaded with one of the three ratiometric techniques, an increase in membrane potential was observed. The increase was slow and continuous without reaching a steady state, even after prolonged incubation. We measured the rate of increase of membrane potential, estimated through the three techniques. Technique I was characterised by an increase of 0.04 ± 0.005 ratiometric units/min (mean ± SD, n = 16; Fig. 4*a*). An increase of 0.06 ± 0.0002 ratiometric units/min was observed with technique II (mean ± SD, n = 11; Fig. 4*b*) and 0.09 ± 0.002 ratio units/min with technique III (mean ± SD, n = 8; Fig. 4*c*).

Discussion

In this study we have tested three techniques for the localisation of mitochondrial and the measurement of the intrinsic activity within these organelles during the initial stages of oogenesis in oocytes of the frog *Xenopus laevis*, where the mitochondrial cloud and several mitochondria are present.

Although hall three techniques involve fluorescence markers as indicators of mitochondrial activity, they differ in the following respects. Rhodamine-123 localises in a charge-dependent manner but is characterised by single-excitation, single-emission fluorescence. Therefore, a second dye is required to enable the differentiation between localisation and mitochondrial activity. In the present experiment we used the dye Mitotracker Red CM-H_2-XRos, which localises in active mitochondrial but does not report membrane potential. The advantage of this technique is the high fluorescence output achieved with rhodamine-123, but

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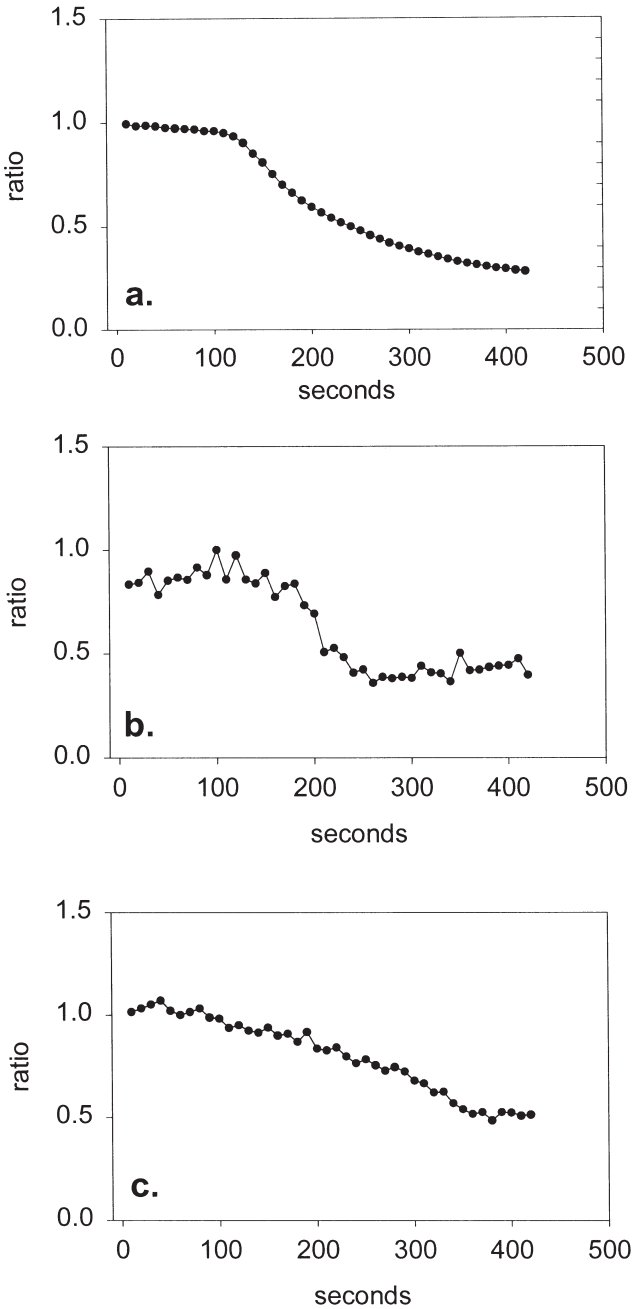

Figure 3 Analysis of the loss of mitochondrial membrane potential after the addition of FCCP. (*a*) Addition of FCCP after loading of frog oocytes with JC-1 (technique I). The figure is a representative example of a total of 6 separate experiments. The ratio is determined as the whole oocyte ratio. FCCP was added to a final concentration of 5 µM at 100 s. (*b*) Addition of FCCP to frog oocytes loaded with rhodamine-123 and Mitotracker Red (technique II). The experiment is a representative example of a total of 14 separate measurements. The ratio measures whole oocyte mitochondrial activity. FCCP was added 5 µM at 100 s. (*c*) Effect of FCCP on frog oocytes loaded with $DiOC_2(3)$ and Mitotracker Green (technique III). The experiment is a representative example of a total of 12 readings. The ratio is measured over the whole oocyte. FCCP was added to 5 µM final concentration at 50 s.

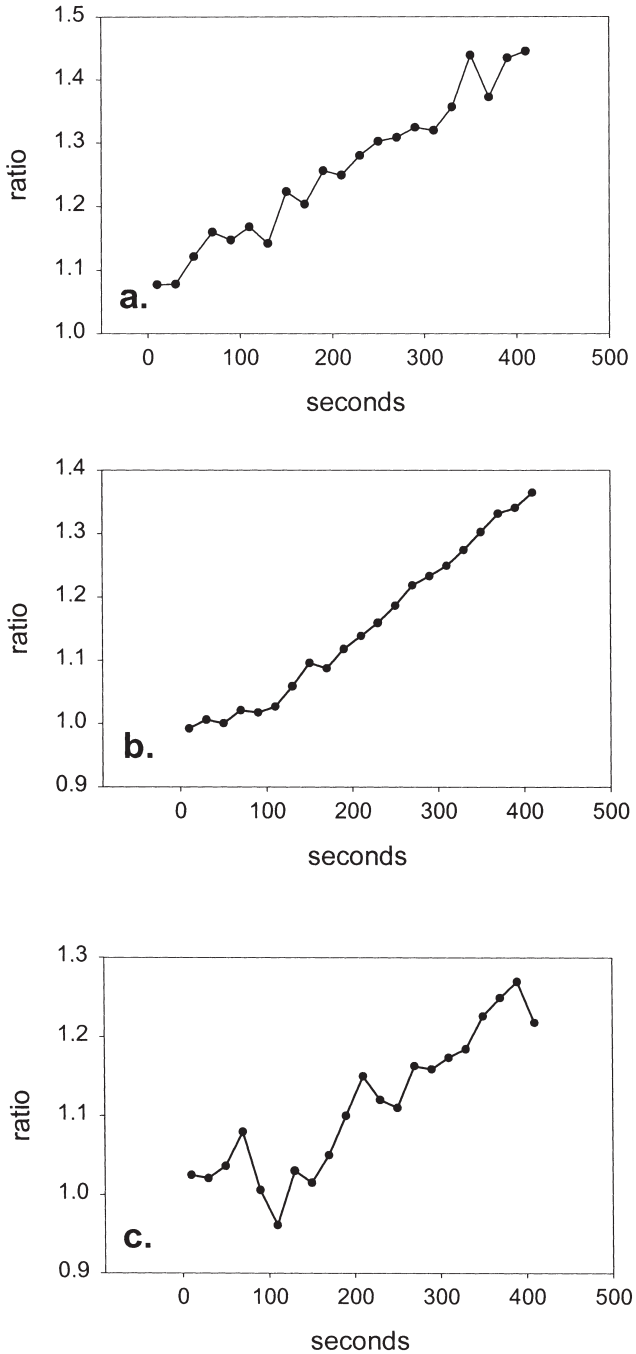

Figure 4 Effect of nigericin on the mitochondrial membrane potential. (*a*) Frog oocytes were loaded with JC-1 (technique I). The experiment shown is a representative example of a total of 16 experiments. Nigericin was added to a final concentration of 5 µM at 20 s. Ratios represent readings of the whole oocyte. (*b*) Frog oocytes were loaded with rhodamine-123 and Mitotracker Red (technique II) and nigericin was added to a final concentration of 5 µM at 50 s. The experiment shown is representative of 11 separate readings. Ratios are of the whole oocyte. (*c*) A representative example of a frog oocyte loaded with $DiOC_2(3)$ and Mitotracker Green (technique III) and challenged with the addition of 5 µM nigericin at 50 s. The experiment is representative of a total of 8 separate experiments. The ratio is of the whole oocyte.

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this is offset by its inability to demonstrate unequivocally that the localisation of the two dyes is equivalent. The $DiOC_2(3)$–Mitotracker Green protocol is characterised by the membrane-potential-dependent localisation of the carbocyanine dye and the localisation of Mitotracker Green as the distribution detector. Again, the disadvantage of this technique is its inability to unequivocally demonstrate that the two dyes localise in equivalent manners. JC-1 is a single-excitation–dual-emission potential-sensitive dye. The huge advantage of this mitochondrial activity marker is the fact that both the potential-sensitive and potential-insensitive fluorescence outputs arise from the same dye, guaranteeing its homogeneous localisation. However, in the present experiments the ratio change between 'inactive' and 'active' mitochondria was the lowest of all three techniques.

Despite these differences, all three techniques gave similar results with respect to mitochondrial activity. Mitochondrial activity was significantly higher within the mitochondrial cloud and in the germinal vesicle region. We noted a 3- to 4-fold greater activity within these zones in all three techniques. These data suggest that mitochondria within the cloud are not only highly localised but also activated. The mechanism of mitochondrial upregulation is not known; however, localised concentrated regions of mitochondrial activity have been observed in a variety of cell types (Smiley *et al.*, 1991). The upregulation of mitochondria in these regions may simply be a result of the increased requirement of ATP, leading to positive feedback on the H^+/ATPase within the mitochondrial matrix.

Treatment of oocytes with the mitochondria uncoupler FCCP caused the rapid dissolution of mitochondrial membrane potential and the corresponding loss of fluorescence from within the mitochondrial cloud. The rate of loss of membrane potential was similar in techniques I and II and significantly slower when technique III was used to measure membrane potential. These data suggest that technique III measures changes within the mitochondrial membrane potential less accurately with respect to the other ratiometric techniques.

In contrast, nigericin, which increases mitochondrial membrane potential through H^+/K^+ exchange, caused an increase in the fluorescence ratio. Although all ratiometric techniques were sufficiently sensitive to measure this ratio, the three techniques gave slightly different results with respect to the rate of change of mitochondrial membrane potential. In fact, techniques I and II again gave similar results, whereas the response measured by technique III was significantly faster. We do not know the reasons for these differences, but the data again suggest that technique III is less accurate than other techniques. We state, therefore, that due to its ease of use and sensitivity in measuring changes in mitochondrial membrane potential, JC-1 should be the dye of choice when examining the mitochondrial membrane potential.

Acknowledgements

This work was supported in part by contributions from the Fondazione Nuovi Orizzonti, Naples, Italy to B. D. We thank Vincenzo Monfrecola for his valuable technical contribution.

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