A calcium signaling defect in the pathogenesis of a mitochondrial DNA inherited oxidative phosphorylation deficiency

MARISA BRINI[1], PAOLO PINTON[2], *MICHAEL P. KING[5], MERCY DAVIDSON[5], ERIC A. SCHON[5,6] & ROSARIO RIZZUTO[2,3,4]

Departments of Biochemistry[1] and Biomedical Sciences[2] and CNR Center for the Study of Biomembranes[3], University of Padova, Italy

[4]Dept. of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Italy

Department of [5]Neurology and of [6]Genetics, College of Physicians and Surgeons of Columbia University, New York, New York, USA

M.P.K. present address: Department of Biochemistry, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

Correspondence should be addressed to R.R.; email: rzr@dns.unife.it

In recent years, genetic defects of the mitochondrial genome (mtDNA) were shown to be associated with a heterogeneous group of disorders, known as mitochondrial diseases[1,2], but the cellular events deriving from the molecular lesions and the mechanistic basis of the specificity of the syndromes are still incompletely understood. Mitochondrial calcium (Ca^{2+}) homeostasis depends on close contacts with the endoplasmic reticulum[3] and is essential in modulating organelle function[4–6]. Given the strong dependence of mitochondrial Ca^{2+} uptake on the membrane potential and the intracellular distribution of the organelle, both of which may be altered in mitochondrial diseases, we investigated the occurrence of defects in mitochondrial Ca^{2+} handling in living cells with either the $tRNA^{Lys}$ mutation of MERRF (myoclonic epilepsy with ragged-red fibers)[7–9] or the ATPase mutation of NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa)[10–13]. There was a derangement of mitochondrial Ca^{2+} homeostasis in MERRF, but not in NARP cells, whereas cytosolic Ca^{2+} responses were normal in both cell types. Treatment of MERRF cells with drugs affecting organellar Ca^{2+} transport mostly restored both the agonist-dependent mitochondrial Ca^{2+} uptake and the ensuing stimulation of ATP production. These results emphasize the differences in the cellular pathogenesis of the various mtDNA defects and indicate specific pharmacological approaches to the treatment of some mitochondrial diseases.

We used cell lines repopulated with mitochondria (and mtDNAs) from patient cytoplasts after depletion of their endogenous mtDNA (ref. 14). We investigated two conceptually different mtDNA mutations. A T→C point mutation at nucleotide 8,356 in the $tRNA^{Lys}$ gene is associated with the disease MERRF (myoclonic epilepsy with ragged-red fibers)[7]. As the MERRF mutation is in a tRNA, it causes a global impairment of mitochondrial protein synthesis, resulting in deficits in both respiratory chain function and in oxidative phosphorylation[8,9]. In contrast, a T→G point mutation at nucleotide 8,993 in the ATPase 6 gene (Leu→Arg at position 156 in the encoded polypeptide) is associated with the disease NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa)[10]. As the NARP mutation is in a specific subunit of ATP synthetase, it impairs only ATP production[11–13], leaving the activity of the respiratory chain relatively unaffected[13]. The assumption underlying our experiments was that a derangement in mitochondrial Ca^{2+} (with possible implications for the control of organelle function) might occur in MERRF, but not in NARP, in which the driving force for Ca^{2+} accumulation should not be affected.

We monitored mitochondrial Ca^{2+} homeostasis with a specifically targeted aequorin chimera, mtAEQ (ref. 15), in cells with 0% and 100% of the MERRF and NARP mutations (Fig. 1). We calibrated the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) from the light output of cells transiently expressing mtAEQ. We placed the cells on the stage of the luminometer and perfused them with Krebs-Ringer buffer, then stimulated them with the inositol 1,4,5 trisphosphate (IP3)-generating agonist histamine. As in HeLa cells, this maneuver induced in control cells a large, transient increase in $[Ca^{2+}]_m$ (peak value, 3.5 ± 0.8 μM; n = 7; Fig. 1*a*, light line), well in the range of the Ca^{2+} sensitivity of the mitochondrial effector systems. In MERRF cells, the $[Ca^{2+}]_m$ increase was reduced considerably (peak value, 1.6 ± 0.6 μM; n = 15; Fig. 1*a*, dark line). The alteration was limited to organelle Ca^{2+} handling. Indeed, when we monitored cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) with an aequorin chimera localized in the cytosol, cytAEQ (ref. 16), we detected no difference between MERRF and control cells either in the basal $[Ca^{2+}]_c$ values or in the amplitude (peak values: MERRF, 1.4 ± 0.6 μM, n = 8; control, 1.5 ± 0.4 μM, n = 10) and kinetics of the agonist-dependent increase (Fig 1*a*, inset). These data indicate that in MERRF cells, the fundamental properties of cytosolic Ca^{2+} homeostasis are not affected. Thus, a cytotoxic Ca^{2+} effect, as reported for the disease of mitochondrial encephalomyopathy, lactic acidosis and stroke-like symptoms[17], is not plausible. Instead, in MERRF cells, the mitochondrial amplification of the cytosolic Ca^{2+} response, a general property of mitochondrial Ca^{2+} homeostasis necessary for the prompt stimulation of the mitochondrial effector systems, seems to be completely lost. This phenomenon was found only with the MERRF cells; in NARP cells, both the mitochondrial and the cytoplasmic Ca^{2+} responses to agonists were indistinguishable from those of control cells (Fig. 1*b*). These data demonstrate a difference in the cellular pathogenesis of the two molecular mtDNA defects that may be a clue to the very different clinical phenotypes in the two disorders.

Two questions remained. The first was whether a therapeutic

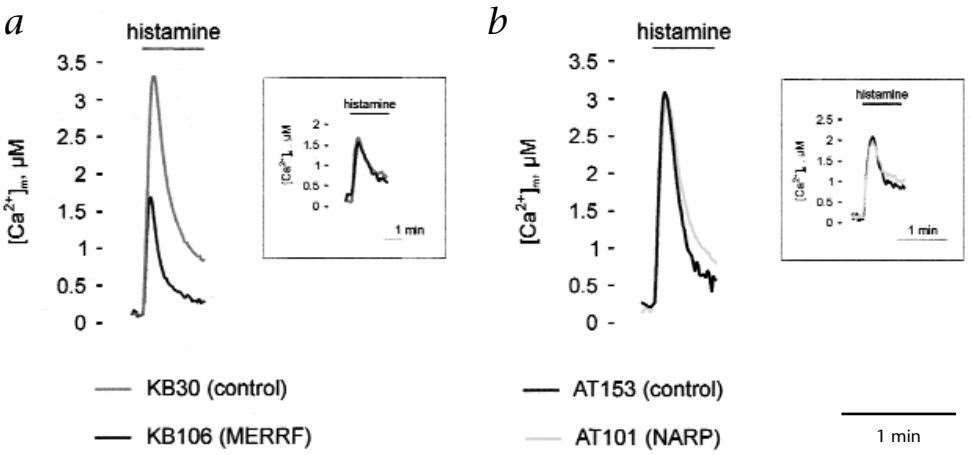

Fig. 1 Histamine-dependent changes in mitochondrial and cytosolic calcium concentrations in MERRF, NARP and control cybrids. $[Ca^{2+}]_m$, mitochondrial; $[Ca^{2+}]_c$, cytosolic (insets). Cells continuously perfused with Krebs-Ringer buffer plus calcium were stimulated with 100 μM histamine. ***a***, MERRF (clone KB106, dark line) and control (clone KB30, light line) cells. ***b***, NARP (clone AT101, light line) and control (clone AT153, dark line) cells. Time scales represent 1 minute. Data are typical of 7–28 experiments, which gave the same results.

approach could be devised for reverting the alteration of mitochondrial Ca^{2+} signaling in MERRF. The agonist-dependent increase in $[Ca^{2+}]_m$ activates the mitochondrial Ca^{2+} antiporters, which, by extruding Ca^{2+} in exchange with Na^+ or H^+, are responsible for the rapid return of $[Ca^{2+}]_m$ to basal values after stimulation[18]. Indeed, in control cells (Fig. 2*a*), the $[Ca^{2+}]_m$ increase evoked by histamine was, in the presence of CGP37157, a specific inhibitor of mitochondrial Ca^{2+} efflux[19], about 20% larger (with CGP37157, 4.1 ± 0.6 μM, n = 8; without CGP37157, 3.5 ± 0.8 μM, n = 7) and more prolonged. This effect was much more profound in MERRF cells, in which uptake is substantially reduced by the impairment of the respiratory chain (and, thus, of the driving force for accumulation). In the presence of CGP37157, the $[Ca^{2+}]_m$ increase evoked by histamine in MERRF cells (Fig. 2*b*) was increased substantially and was prolonged, approaching the peak amplitudes in control cells (MERRF, 3.1 ± 0.6 μM, n = 15; control, 3.5 ± 0.8 μM, n = 7; Fig. 2*a*, light line, and *b*, dark line). The effect on cytosolic Ca^{2+} handling was negligible (peak values: untreated, 1.4 ± 0.6 μM, n = 8; treated, 1.3 ± 0.4 μM, n = 11; Fig. 2*b*, inset), providing an example of the selectivity and usefulness of an organelle-specific Ca^{2+} drug. Finally, in NARP cells (Fig. 2*c*) CGP37157 caused an increase in the $[Ca^{2+}]_m$ peak similar to that in control cells (about 20%).

The second question was whether the restoration of mitochondrial Ca^{2+} homeostasis had any beneficial effect on the pathological phenotype. To investigate this, we monitored mitochondrial ATP levels with a specifically targeted luciferase chimera mtLUC (L.S. Jouaville, *et al.*, manuscript submitted). This new approach is based on the observation that, in the cellular environment and in the presence of luciferin, luciferase light emission is a linear function of ATP concentration in the physiological range[20,21]. In HeLa cells, the agonist-dependent mitochondrial Ca^{2+} signal is the direct trigger of the activation of mitochondrial ATP production (L.S. Jouaville *et al.*, manuscript submitted). In agreement with these data, in control cells, histamine stimulation caused a large increase (+43 ± 12%; n = 15) in

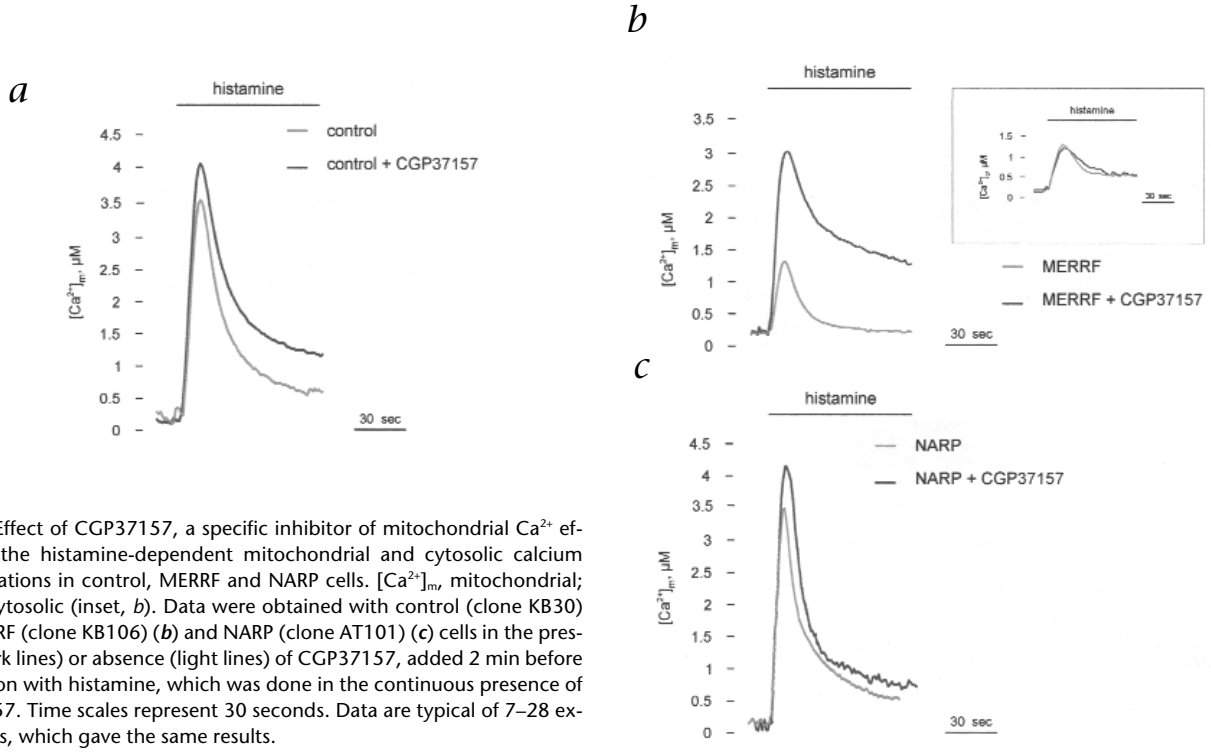

Fig. 2 Effect of CGP37157, a specific inhibitor of mitochondrial Ca^{2+} efflux, on the histamine-dependent mitochondrial and cytosolic calcium concentrations in control, MERRF and NARP cells. $[Ca^{2+}]_m$, mitochondrial; $[Ca^{2+}]_c$, cytosolic (inset, *b*). Data were obtained with control (clone KB30) (***a***), MERRF (clone KB106) (***b***) and NARP (clone AT101) (***c***) cells in the presence (dark lines) or absence (light lines) of CGP37157, added 2 min before stimulation with histamine, which was done in the continuous presence of CGP37157. Time scales represent 30 seconds. Data are typical of 7–28 experiments, which gave the same results.

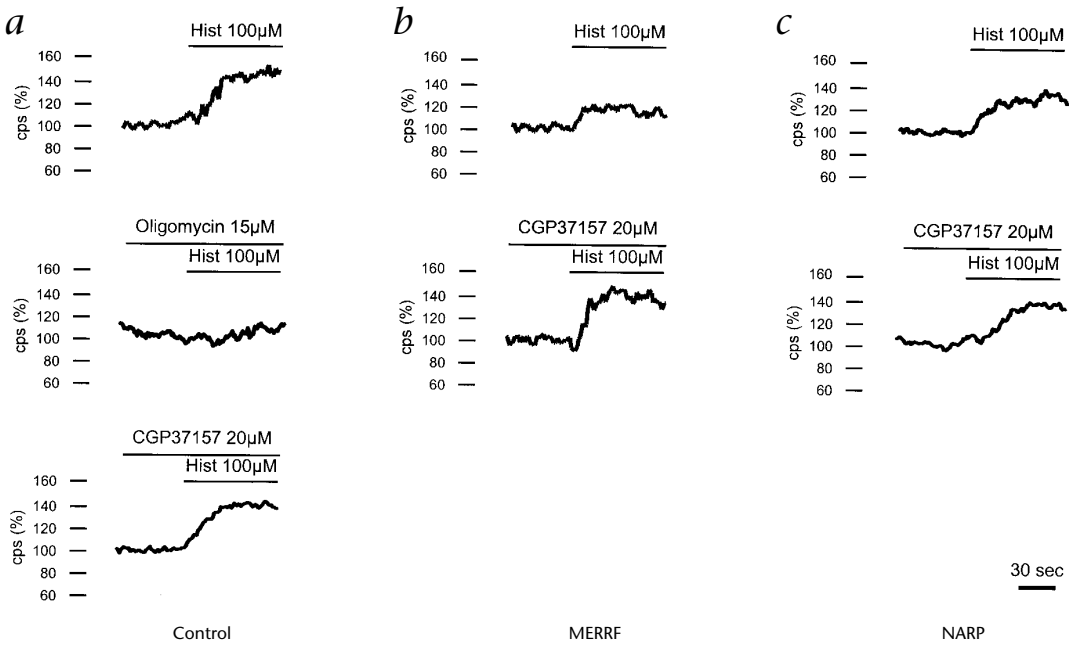

Fig. 3 Agonist-dependent stimulation of mitochondrial ATP production in control, MERRF and NARP cells. Data are expressed as percent of mtLUC light output of cells before agonist stimulation. ***a***, Control cells (clone KB30). ***b***, MERRF cells (clone KB106). ***c***, NARP cells (clone AT101). Above traces, cells were treated with 100 μM histamine (Hist), 15 μM oligomycin and/or 20 μM CGP37157. cps, counts per second. Data are typical of 7–28 experiments, which gave the same results.

mitochondrial ATP levels (Fig. 3*a*). The increase was completely abolished by pre-treatment with the ATPase inhibitor oligomycin, indicating that it is entirely attributable to ATP synthesis and not to an effect on luciferase light emission by changes in matrix pH occurring after mitochondrial activation. For the absolute ATP concentrations, we calibrated the luciferase signal by permeabilizing the cells at the end of the experiment and perfusing them with an 'intracellular' buffer, supplemented with 100 μM EGTA and known concentrations of ATP. We obtained an estimate of about 5 mM for the resting ATP concentration; however, differences in the availability of luciferin may introduce a substantial error in extrapolating the values occurring in intact cells. Finally, pre-treatment with CGP37157, which augments the histamine-dependent $[Ca^{2+}]_m$ peak (Fig. 2*a*), did not enhance the agonist-dependent increase (+47 ± 8%; n = 10) in the mitochondrial ATP levels. In MERRF cells, whereas the calibrated ATP concentration estimate was the same as in control cells (about 5 mM), the agonist-dependent increase was reduced considerably (+10 ± 5%; n = 15) (Fig. 3*b*). Pretreatment of MERRF cells with CGP37157, by greatly augmenting the amplitude of the $[Ca^{2+}]_m$ increase (Fig. 2*b*), mostly restored the histamine-dependent increase in ATP levels (+39 ± 8%; n = 20).

The driving force for both ATP synthesis and Ca^{2+} uptake is provided by the proton electrochemical gradient established by the respiratory chain, so both processes are expected to be affected, to some extent, in MERRF cells. Our data indicate that the impairment of mitochondrial Ca^{2+} signaling is essential. This is not entirely unexpected, given that Ca^{2+} transfer from the endoplasmic reticulum to the mitochondria is temporally limited to the early phase of Ca^{2+} release, when the high concentrations of Ca^{2+} at the 'mouth' of the IP3-gated channels meet the low affinity of the mitochondrial Ca^{2+} uniporter[3]. Thus, the MERRF mutation, by reducing the driving force for Ca^{2+} accumulation, interferes substantially with this highly transient phenomenon. The defective signaling to mitochondria of the higher requirements of a stimulated cell directly effects the energy levels of the cell, as shown by the direct monitoring of cytoplasmic ATP with a targeted luciferase chimera (cytLUC) (data not shown). After correction of the signaling defect, the diseased mitochondria, despite the lower proton gradient, can still enhance their ATP production.

Finally, compared with control cells, NARP cells showed a small reduction in the histamine-dependent increase in the mitochondrial ATP levels (NARP (AT101), +29 ± 8%, n = 28; control (AT153), +35 ± 10%, n = 20). As in control cells, pre-treatment with CGP37157 did not enhance (+34 ± 9%, n = 15) the histamine-dependent increase in ATP levels (Fig. 3*c*).

Thus, our investigation of Ca^{2+} signaling in matched pairs of transmitochondrial cell lines with two different mtDNA mutations showed a distinct difference in the cellular events deriving from the molecular defects. We demonstrated that for partial deficiencies of the respiratory chain (that is, in the MERRF $tRNA^{Lys}$ mutation, and presumably also in other mutations that impair global protein synthesis, such as the mtDNA deletions), there is a large derangement of organellar Ca^{2+} handling, which impairs the calcium-mediated activation of mitochondrial activity. As a consequence, mitochondrial (and cytosolic) ATP levels reduced by the ATP-consuming processes occurring in the cytosol of a stimulated cell are not restored rapidly. Specific drugs acting on mitochondrial Ca^{2+} handling restored both the Ca^{2+} signal and the enhancement of ATP production, which emphasizes the importance of cell biology studies for the understanding of the pathogenesis of mitochondrial diseases, and may open the way to new biochemically designed therapeutic approaches to treat these disorders.

Methods

Transmitochondrial cell lines. A human ρ° osteosarcoma line (143B206) completely devoid of endogenous mtDNA was generated by treatment of the parental ρ⁺ osteosarcoma line (143B) with ethidium bromide, as described[14]. Transmitochondrial cell lines (cybrids) containing 100% wild-type mtDNA (KB30) and 100% mutant mtDNA (KB106) were then generated by fusing 143B206 cells with cytoplasts derived from heteroplasmic cells obtained from a patient with the MERRF-T8356C mutation, as described[9]. The same methods were also used to generate cybrids containing 100% wild-type mtDNA (AT153) and 100% mutant mtDNA (AT101) from heteroplasmic fibroblasts obtained from a subject with the NARP-T8993G mutation. Homoplasmy of mtDNA genotype within each cybrid line was confirmed by

PCR–RFLP analysis, using appropriate flanking PCR primers and restriction sites diagnostic for the presence or absence of the respective mutations[7,9,10].

Cell cultures and transfection. KB30, KB106, AT101 and AT153 cells were grown in 75-cm^2 flasks with Dulbecco's modified Eagle's medium (4,500 mg glucose/liter and 110 mg pyruvate/liter), supplemented with 10% fetal calf serum, 2 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin. For transient expression experiments, the cells were seeded onto coverslips 13 mm in diameter and allowed to grow to 50% of confluence. At this stage, calcium phosphate transfection with the aequorin and luciferase expression plasmids (4 μg DNA/coverslip) was done as described[22]. Aequorin and luciferase measurements were made 24 h after transfection.

Aequorin measurements. At 24 h after transfection, transfected aequorin was reconstituted by incubating the cells for 1–3 h with 5 μM coelenterazine in DMEM supplemented with 1% fetal calf serum in a 5% CO_2 atmosphere. The coverslips with the cells were then transferred to a perfused chamber with a thermostat placed in close proximity to a low-noise photomultiplier, with a built-in amplifier–discriminator. The output of the discriminator was captured by a Thorn EMI photon counting board and stored in a IBM-compatible computer for further analysis[22]. During the experiments, the cells were perfused with modified Krebs-Ringer buffer (125 mM NaCl, 5 mM KCl, 1mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4 at 37 °C), supplemented with 1 mM $CaCl_2$ and challenged with agonists and drugs added to the same medium. CGP37157 (20 μM) was a gift from Ciba-Geigy. At the end of the experiment, the cells were lysed by perfusing them with a 10-mM $CaCl_2$ solution, to discharge unconsumed aequorin and estimate the total photoprotein content. Aequorin photon emission was then calibrated offline into $[Ca^{2+}]$ values, using a computer algorithm based on the Ca^{2+} response curve of wild-type aequorin, as described[16].

Luciferase measurements. Cell luminescence was measured in the same luminometer used for the aequorin measurements. Cells were constantly perfused with Krebs-Ringer buffer, supplemented with 1 mM $CaCl_2$ and 20 μM luciferin. All additions were made to this medium. The light output of a coverslip of transient trasfected cells was 1,000–10,000 counts per second (cps), compared with a background output of less than 10 cps. All compounds used in the experiments were tested for nonspecific effects on the luminescence and none were found. To obtain an estimate of the absolute ATP concentrations, digitonin-permeabilized cells were perfused with an 'intracellular' buffer (140 mM KCl, 5 mM NaCl, 1 mM K_3HPO_4, 2 mM $MgSO_4$, 20 mM Hepes, pH 7.05 at 37 °C), supplemented with 100 μM EGTA, 20 μM luciferin and known concentrations of ATP and $MgCl_2$.

Acknowledgments

We thank T. Pozzan and P. Magalhães for criticism and discussion, and G. Ronconi, M. Santato, B. Filiano and E. Davidson for technical assistance. The work was supported by funds from 'Telethon' (project number 850) and from the Italian University and Health Ministries to R.R., and grants from the National Institutes of Health (NS28828, NS11766 and HD32062) and the Muscular Dystrophy Association of USA to E.A.S. and M.P.K.

RECEIVED 10 MAY; ACCEPTED 16 JUNE 1999

1. Schon, E.A., Bonilla, E. & DiMauro, S. Mitochondrial DNA mutations and pathogenesis. *J. Bioenerg. Biomembr.* **29**, 131–149 (1997).
2. Wallace, D.C. Mitochondrial DNA mutations in diseases of energy metabolism. *J. Bioenerg. Biomembr.* **26**, 241–50 (1994).
3. Rizzuto, R. *et al.* Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280**, 1763–1766 (1998).
4. McCormack, J.G., Halestrap, A.P. & Denton, R.M. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol. Rev.* **70**, 391–425 (1990).
5. Hansford, R.G. Physiological role of mitochondrial Ca^{2+} transport. *J. Bioenerg. Biomembr.* **26**, 495–508 (1994).
6. RobbGaspers, L. D. *et al.* Integrating cytosolic calcium signals into mitochondrial metabolic responses. *EMBO J.* **17**, 4987–5000 (1998).
7. Silvestri, G. *et al.* A new mtDNA mutation in the tRNALys gene associated with myoclonic epilepsy and ragged-red fibers (MERRF). *Am. J. Hum. Genet.* **51**, 1213–1217 (1992).
8. Enriquez, J.A., Chomyn, A. & Attardi, G. MtDNA mutation in MERRF syndrome causes defective aminoacylation of tRNA(Lys) and premature translation termination. *Nature Genet.* **10**, 47–55 (1995).
9. Masucci, J. *et al. In vitro* analysis of mutations causing myoclonus epilepsy with ragged-red fibers in the mitochondrial tRNALys gene: two genotypes produce similar phenotypes. *Mol. Cell. Biol.* **15**, 2872–2881 (1995).
10. Holt, I.J. *et al.* A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. *Am. J. Hum. Genet.* **46**, 428–433 (1990).
11. Tatuch, Y. & Robinson, B.H. The mitochondrial DNA mutation at 8993 associated with NARP slows the rate of ATP synthesis in isolated lymphoblast mitochondria. *Biochem. Biophys. Res. Comm.* **192**, 124–128 (1993).
12. Houstek, J. *et al.* Altered properties of mitochondrial ATP-synthase in patients with a T->G mutation in the ATPase 6 (subunit a) gene at position 8993 of mtDNA. *Biochim. Biophys. Acta* **1271**, 349–357 (1995).
13 Vazquez-Memije, M.E. *et al.* Comparative biochemical studies in fibroblasts from patients with different forms of Leigh syndrome. *J. Inher. Metab. Dis.* **19**, 43–50 (1996).
14 King, M.P. & Attardi, G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**, 500–503 (1989).
15. Rizzuto, R. *et al.* Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature* **358**, 325–328 (1992).
16. Brini, M. *et al.* Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$): a critical evaluation. *J. Biol. Chem.* **270**, 9896–9903 (1995).
17. Moudy, A.M. *et al.* Abnormal calcium homeostasis and mitochondrial polarization in a human encephalomyopathy. *Proc. Natl. Acad. Sci. USA* **92**, 729–733 (1995).
18. Rizzuto, R. *et al.* Mitochondrial Ca^{2+} homeostasis in intact cells. *J. Cell Biol.* **126**, 1183–1194 (1994).
19. Cox, D.A. & Matlib, M.A. A role for the mitochondrial Na^{+}-Ca^{2+} exchanger in the regulation of oxidative phosphorylation in isolated heart mitochondria. *J. Biol. Chem.* **268**, 938–947 (1993).
20. Allue, I. *et al.* Evidence for rapid consumption of millimolar concentrations of cytoplasmic ATP during rigor-contracture of metabolically compromised single cardiomyocytes. *Biochem. J.* **319**, 463–469 (1996).
21. Maechler, P., Wang, H. & Wollheim, C. B. Continuous monitoring of ATP levels in living insulin secreting cells expressing cytosolic firefly luciferase. *FEBS Lett.* **422**, 328–332 (1998).
22. Rizzuto, R. *et al.* Photoprotein mediated measurement of $[Ca^{2+}]$ in mitochondria of living cells. *Meth. Enzymol.* **260**, 417–428 (1995).