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Influence of a mitochondrial genetic defect on capacitative calcium entry and mitochondrial organization in the osteosarcoma cells

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Abstract **Effects of T8993G mutation in mitochondrial DNA (mtDNA), associated with neurogenical muscle weakness, ataxia and retinitis pigmentosa (NARP), on the cytoskeleton, mitochondrial network and calcium homeostasis in human osteosarcoma cells were investigated. In 98% NARP and ρ^0 (lacking mtDNA) cells, the organization of the mitochondrial network and actin cytoskeleton was disturbed. Capacitative calcium entry (CCE) was practically independent of mitochondrial energy status in osteosarcoma cell lines. The significantly slower Ca^{2+} influx rates observed in 98% NARP and ρ^0, in comparison to parental cells, indicates that proper actin cytoskeletal organization is important for CCE in these cells.**

Keywords: Osteosarcoma; Mitochondria; Cytoskeleton; Capacitative calcium entry; NARP

1. Introduction

Mitochondria are unique organelles in their double genetic origin. Eleven essential subunits of the respiratory chain and two subunits of ATP synthase are encoded in mitochondrial DNA (mtDNA). Unlike nuclear genes, mitochondrial genes are present in high copy numbers. Most mtDNA alterations can lead to human disorders [1,2] and usually wild type and mutant mtDNA coexist in the cells of patients with mtDNA diseases.

Neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP), one of the common human mitochondrial diseases, can be the result of the mtDNA mutation T8993G in ATP6 gene encoding subunit 6 of the mitochondrial ATP synthase. NARP develops when the level of T8993G mutation exceeds 70% penetration. At an extreme degree of heteroplasmy (over 90%), the T8993G mutation can cause fatal Leigh syndrome. The T899G mtDNA mutation is associated with a decreased capacity of ATP synthesis [3–6].

ρ^0 cells that lack mtDNA have been used widely to study the importance of mtDNA for cell functions. These cells show a characteristic lack of mitochondrially encoded protein subunits, resulting in a disruption of respiratory function and initiating cellular energy production by glycolysis [7]. These cells cannot carry out oxidative phosphorylation and contain swollen mitochondria, which still import and process most proteins encoded by nuclear DNA. Buchet et al. [8] have shown that F_1-ATPase and the adenine nucleotide translocator are functional in ρ^0 cells and are able to generate, at least to some extent, mitochondrial membrane electric potential.

Proper intracellular distribution of mitochondria is essential for normal cell function and the functionality of mitochondria is reflected in their structure [9,10]. Confocal imaging of live cells shows a continuous reorganization of mitochondrial structures [10–13]. Rapid changes in mitochondrial morphology appear to be controlled by a dynamic balance between cytoskeletal organization and fission/fusion events [11,14,15] and alterations in mitochondrial morphology have been correlated with changes of mitochondrial metabolic state [15–19]. An increasing body of evidence indicates that mitochondrial network dynamics is important for many aspects of cellular function. Moreover, the importance of cytoskeletal proteins as modulators of cell morphology and signaling has inspired speculations that mitochondrial network dynamics and regulation of intracellular calcium levels are closely related [9,20–22]. Elevated cytosolic calcium concentration ($[Ca^{2+}]_c$) is another obvious candidate for causing cellular dysfunction in mitochondrial diseases. Mitochondria could transiently accumulate an appreciable amount of calcium and thereby affect calcium homeostasis. This function is related to the rapid uptake and relatively slow release of calcium ions by mitochondria. Mitochondria localized in regions close to the endoplasmic reticulum (ER) and plasma membrane (PM) are most effective in $[Ca^{2+}]_c$ buffering [9,23]. In electrically non-excitable cells, calcium influx into the cell depends on the filling state of ER. Mitochondria are postulated to play an important role in the regulation of capacitative calcium entry (CCE) [24–26]. Recently, Zabłocki et al. [27] have shown that extracellular pH affects CCE regulation in Jurkat cells. Deenergization of mitochondria greatly impairs the activity of CCE even under conditions of full calcium release from the ER [28]. Mutations in mtDNA can also result in reduction of calcium influx [29–31], but do not influence the calcium content in mitochondria in NARP cells [32].

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Abbreviations: $[Ca^{2+}]_c$, cytosolic calcium concentration; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CCE, capacitative calcium entry; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide; mtDNA, mitochondrial DNA; MTs, microtubules; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; $\Delta\Psi$, mitochondrial membrane electric potential; PM, plasma membrane

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The aim of this study was to characterize changes in mitochondrial and cytoskeletal organization caused by various levels of penetration of the mitochondrial T8993G mutation. We have studied the morphology of mitochondria as well as the distribution of actin, microtubules and vinculin in cells with different degrees of T8993G penetration. We also determined how the penetration of mtDNA T8993G mutation influences CCE. We have compared the rate of calcium influx in osteosarcoma cell lines before and after treatment with the protonophore CCCP and have demonstrated that mitochondria in osteosarcoma cells do not control the rate of Ca^{2+} flux through the PM.

2. Materials and methods

2.1. Materials

Fura-2/AM, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1), MitoTracker CMXRos and rhodamine phalloidin were from Molecular Probes (Eugene, OR). Thapsigargin was purchased from Calbiochem (La Jolla, CA). Fluorescently labeled anti-mouse and anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-tubulin antibody was from BD Biosciences Pharmingen (Lexington, KY). Other chemicals and antibodies were from Sigma Chemicals (St. Louis, MO).

2.2. Cell lines and culture conditions

The parental human osteosarcoma cell line 143B (TK^-), its 143B/206 cybrids with different degrees of penetration (60% and 98%) of the mtDNA T8993G mutation, and the ρ^0 cell line lacking mtDNA, derived from NARP skin fibroblasts, were prepared according to the methods of King and Attardi [7] and were kindly provided by Dr. M. Tanaka from the Department of Gene Therapy, Gifu International Institute of Biotechnology, Japan.

Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (GIBCO BRL), supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL), antibiotics such as 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM pyruvate and uridine 50 μg/ml [33]. All experiments were performed on cells between 2 and 4 passages.

2.3. Immunocytochemical staining

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed with 10% FBS/PBS, and incubated for 1 h with primary antibodies in 10% FBS/PBS supplemented with 0.2% saponin. After washing three times with 10% FBS/PBS to remove unbound antibody, the cells were incubated for 1 h with the appropriate fluorescently conjugated secondary antibodies diluted in 10% FBS/PBS containing 0.2% saponin. Coverslips were washed three times with 10% FBS/PBS, once with PBS, and then mounted on a slide.

2.4. Mitochondrial imaging

To visualize changes in morphology of mitochondria, the cells were incubated with 100 nM MitoTracker CMXRos [34] in the dark at 37 °C for 10 min. The cells were then briefly rinsed with the medium, incubated with all experimental reagents (CCCP, oligomycin and thapsigargin) under the same conditions (temperature, time of incubation and concentration) as for $[Ca^{2+}]_c$ measurements, and fixed. CMXRos is a cationic lipophilic dye, preferentially sequestered into mitochondria and reacting with thiols of proteins in the mitochondrial matrix.

2.5. Mitochondrial membrane potential

Mitochondrial membrane potential was measured using JC-1 [35–37]. This compound is a lipophilic cationic dye that accumulates in the mitochondrial matrix proportionally to electric potentials across the inner mitochondrial membrane. At higher concentrations, JC-1 forms aggregates. Fluorescence (excitation max. 490 nm) of the monomer is green (emission max. 527 nm), whereas that of the aggregate is red (emission max. 590 nm).

Cells grown on coverslips were incubated in the dark for 15 min at 37 °C with the medium containing freshly prepared 3 μM JC-1 and then washed three times with the medium without JC-1. The cells were observed immediately after labeling and for no more than 20 min. Confocal microscopy was performed at room temperature. Background images were obtained from a cell-free section of the coverslip. A ratio image generated by dividing the fluorescence intensity at 590 nm by the fluorescence intensity at 527 nm is reported as a relative mitochondrial membrane electric potential ($\Delta\Psi$) value.

2.6. Confocal immunofluorescence microscopy and image analysis

Fluorescence microscopy was carried out using a Leica TCS SP2 Spectral Confocal and Multiphoton Microscope with 63× oil immersion objective. Images were acquired from randomly selected fields of non-confluent cells.

Imaging conditions (gain levels, confocal aperture size and laser power) were held constant in a series of images and the fast scan option was used to minimize bleaching and phototoxic effects. To quantify changes in fluorescence intensities of particular cell areas, at least five areas (about 100 cells in each area) were selected.

2.7. $[Ca^{2+}]_c$ measurements

The standard medium consisted of 130 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 1 mM pyruvate and 5 mM glucose; pH (7.2 or 7.8) was adjusted to the desired value with NaOH. Where indicated: 0.12 μM oligomycin, 2 μM CCCP and 100 nM thapsigargin were added as DMSO solutions. In control samples, CCCP was replaced by DMSO alone.

Cytosolic free Ca^{2+} was measured with the fluorescent probe Fura-2 [38]. Cells were loaded with this probe by incubation in the culture medium supplemented with 1 μM Fura-2/AM at 37 °C for 15 min. After washing the cells, the coverslip was placed in a cuvette filled with the standard, nominally calcium-free, medium (supplemented with 50 μM EGTA). Thapsigargin, oligomycin, CCCP and 3 mM $CaCl_2$ were added as indicated and $[Ca^{2+}]_c$ was monitored fluorimetrically. Fluorescence was measured at 30 °C in a Shimadzu RF5000 fluorimeter set in the ratio mode using 340 nm/380 nm as the excitation and 510 nm as the emission wavelengths. Time resolution of the measurement was 1 s. $[Ca^{2+}]_c$ was calibrated for cells in each run using 3 mM of externally added $CaCl_2$ and 3 μM ionomycin or digitonin (final concentration 0.001%).

2.8. Data analysis

Data presented are means ± S.D. of at least three different experiments.

3. Results

3.1. Organization of mitochondria in normal and mtDNA-mutated cells

Mitochondrial genetic defects may modify the organization of this organelle within the cell [17,39]. The morphology and organization of mitochondria were studied using the dye MitoTracker CMXRos. In the parental and 60% NARP cybrid cell lines, most of the mitochondria formed elongated, continuous tubular structures, which had the appearance of branched reticulum filaments distributed throughout the cell (Figs. 1A and B). In the same field, we also observed a few cells with single, round-shaped mitochondria. A quite different mitochondrial organization was seen in the 98% NARP and ρ^0 cell lines. In these cells, the mitochondrial reticulum appeared to be disrupted, forming numerous isolated organelles (Figs. 1C and D).

Thapsigargin (a selective inhibitor of Ca^{2+}-ATPase in ER membrane) or oligomycin (an inhibitor of mitochondrial

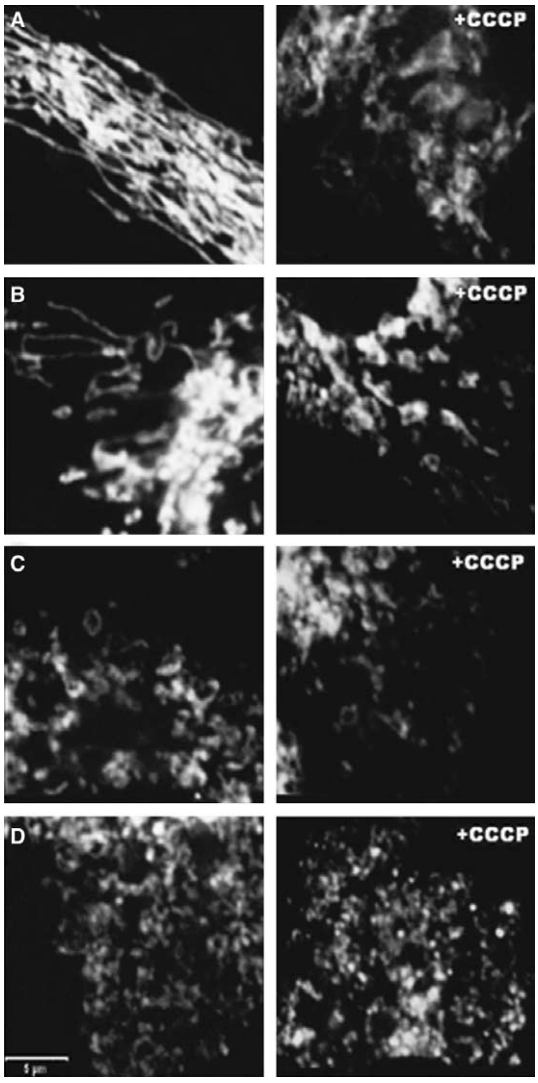

Fig. 1. Visualization of mitochondria by mitoTracker CMXRos in: (A) parental osteosarcoma cell lines; (B) 60% NARP cybrid cells; (C) 98% NARP cybrid cells; and (D) ρ^0 cells.

F_1F_O-ATPase) added to cells (time of incubation and concentrations identical as for $[Ca^{2+}]_c$ measurements) did not modify mitochondrial morphology. On the other hand, mitochondrial uncoupler CCCP (2 μM) added to parental osteosarcoma and 60% NARP cells caused disorganization of the filamentous network within 2 min (Fig. 1).

3.2. Cytoskeletal organization of normal and mtDNA-mutated cells treated and untreated with CCCP

Mitochondria are known to move along microfilaments and MTs cytoskeletal tracks. Confocal images revealed that MTs formed bundles with mitochondria distributed along them. The organization of MTs was the same in all investigated cell lines. Incubation with thapsigargin and oligomycin plus CCCP did not influence the MT network within the cell.

In parental osteosarcoma cells, F-actin formed a well-organized microfilament network with strong cortical actin filaments (Fig. 2). A similar actin organization was found in 60% NARP cybrid cells (not shown). In contrast, actin filaments in ρ^0 cells were uniformly distributed throughout the

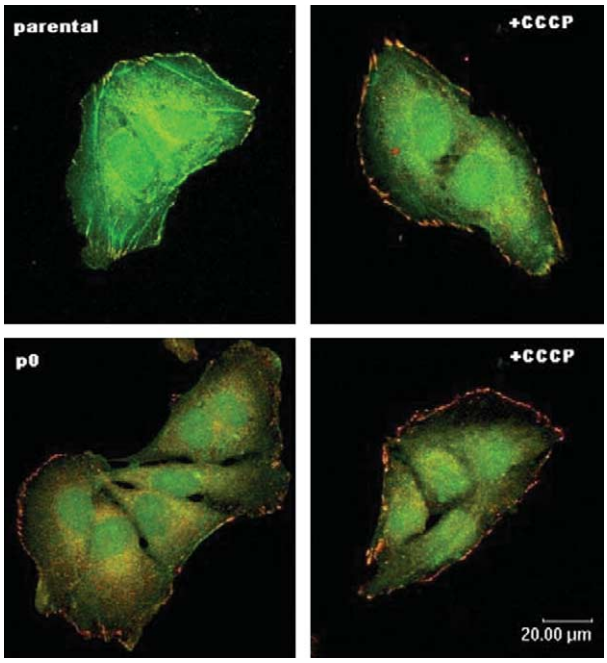

Fig. 2. Actin filaments and vinculin organization in the parental and ρ^0 cells before and after incubation with CCCP (2 μM, 2 min). Actin was labeled with phalloidin-FITC (green) and vinculin with monoclonal anti-vinculin and TRITC-conjugated secondary antibody (red).

cytoplasm and very few actin filaments were found close to the PM (Fig. 2). The same organization of actin filaments as in ρ^0 cells was found in 98% NARP cybrid cells (not shown). Treatment of cells with CCCP substantially reduced the amount of F-actin at the cell cortex in all cell lines (Fig. 2).

Vinculin distribution differed slightly in the different osteosarcoma cell lines. ρ^0 cells showed a higher accumulation of vinculin at the cell edge compared to parental control cells (Fig. 2). The organization of vinculin in 60% NARP cells was the same as in parental cells, but 98% NARP cells were almost identical to ρ^0 cells.

3.3. Effects of T8993G mtDNA mutation and mtDNA depletion on mitochondrial membrane electric potential

The morphology of different cell lines and the ability of mitochondria to generate $\Delta\Psi$ were simultaneously monitored. Fig. 3 shows the extent to which mitochondria in the osteosarcoma cell lines can generate $\Delta\Psi$. ρ^0 cells contain mitochondria with relatively low $\Delta\Psi$. Mitochondria in 60% NARP and 98% NARP cybrid cells could generate $\Delta\Psi$ of the same magnitude as parental cell mitochondria. Quantitative estimation of $\Delta\Psi$ with JC-1 is consistent with the observations shown in Fig. 4. There were many JC-1 aggregates (red fluorescence) in mitochondria in the parental cells, but such aggregates are nearly absent in ρ^0 cells. Moreover, JC-1 fluorescence showed coexistence of two populations of mitochondria, one with high $\Delta\Psi$ (red) and one with low $\Delta\Psi$ (green). In parental cells, filamentous mitochondria, which stained green, occurred throughout the cytoplasm, whereas mitochondria with intense red fluorescence were found mainly near the PM. In ρ^0 cells, granular mitochondria emitted green fluorescence, indicating that a low $\Delta\Psi$ was established across their inner membrane (Fig. 4).

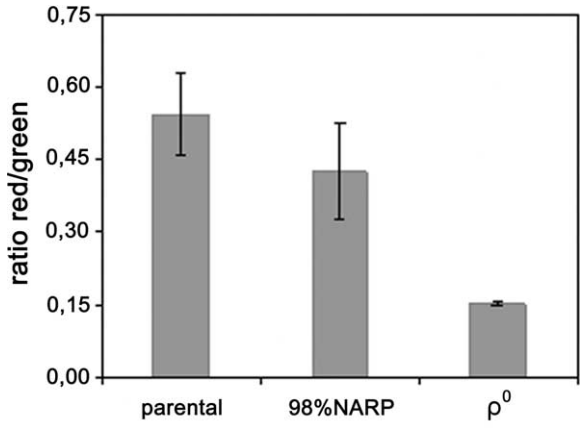

Fig. 3. JC-1 fluorescence ($\Delta\Psi$) in human osteosarcoma parental cells, 98% NARP cybrid cells and ρ^0 cells. Data from one experiment out of 3 are presented.

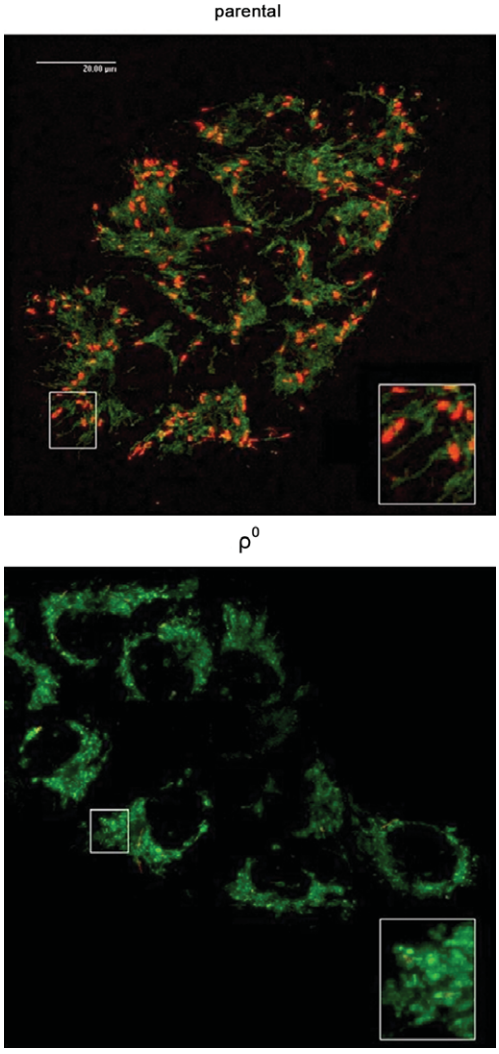

Fig. 4. Accumulation of JC-1 by mitochondria within parental and ρ^0 cells visualized by confocal laser fluorescence microscopy. All pictures were taken with the same exposure time. The insets show magnified images of the equivalent areas in each JC-1 image indicated by squares. Red, aggregated JC-1; green, monomeric JC-1.

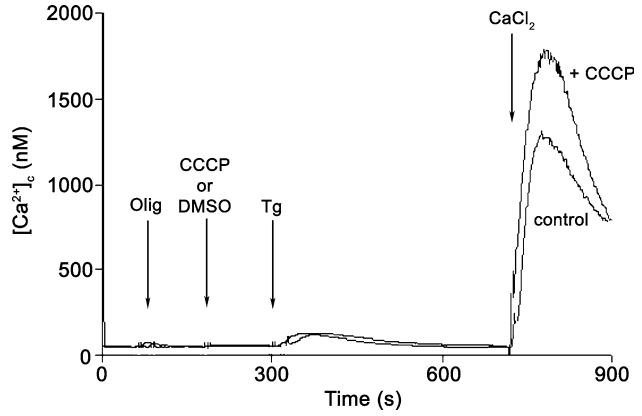

Fig. 5. $[Ca^{2+}]_c$ changes in parental osteosarcoma cells. The effect of CCCP. The cells were loaded with Fura-2 and incubated in the standard calcium-free medium. Where indicated, 0.12 μM oligomycin, 2 μM CCCP, 0.1 μM thapsigargin and 3 mM $CaCl_2$ were added. Typical traces from 10 experiments are presented.

3.4. Effects of defective mitochondria on the calcium influx

The addition of thapsigargin to cells incubated in Ca^{2+}-free media produced a $[Ca^{2+}]_c$ transient, initially due to the release of calcium ions from the ER (the phase of increase in $[Ca^{2+}]_c$) and then to pumping of Ca^{2+} out of cells by PM calcium-ATPase (the phase of return to the basal $[Ca^{2+}]_c$). Subsequent addition of 3 mM $CaCl_2$ to the extracellular medium produced a rapid flow of Ca^{2+} into the cell and a corresponding sustained enhancement of $[Ca^{2+}]_c$. The initial rates of Ca^{2+} fluxes into investigated osteosarcoma cell lines were: 1.02 ± 0.18, 0.50 ± 0.06 and 0.59 ± 0.11 mM/min, for the parental, 98% NARP and ρ^0 cells, respectively. In addition, pretreatment of the parental cells with oligomycin plus CCCP did not influence the rate of calcium flux into thapsigargin-pretreated cells, triggered by $CaCl_2$ addition (Fig. 5). The same observation was made in the case of other investigated osteosarcoma cell lines (not shown).

4. Discussion

4.1. Characterization and comparison of mitochondrial network in the osteosarcoma cell lines

Apart from the abnormalities in assembly of ATP synthase and decrease of ATP synthesis capacity, there is little information about the phenotype of NARP mutant cells. The results presented in this paper clearly demonstrate that there are differences in mitochondrial organization in cells with high T8993G mtDNA penetration. In parental osteosarcoma cells and in 60% NARP cells, mitochondria exist predominantly as a tubule-like organized network (Figs. 1 and 4). A similar mitochondrial network has been described in many wild-type cell lines [9,17,20]. On the other hand, swollen and fragmented mitochondria were observed in 98% NARP and in ρ^0 cells.

It seems that a single parental cell contains heterogeneous mitochondria with respect to $\Delta\Psi$ (Fig. 4). This heterogeneity may reflect discrete roles of different mitochondria depending on their localization within the cytosol. Collins et al. [9] have shown that peripheral mitochondria accumulate substantially more Ca^{2+} than those in the perinuclear region. Perhaps, peripheral mitochondria sequester Ca^{2+} entering through CCE, whereas perinuclear mitochondria may be important in

regulating bulk phase calcium signals. Thus, various subpopulations of mitochondria sequester Ca^{2+} from different sources. Mitochondria at different localizations in the cytosol can be activated by calcium ions to different degrees [13,39].

In the present paper, in agreement with [35], we also demonstrated a lower level of energized mitochondria in ρ^0. In contrast, Garcia et al. [40] reported that in NARP and ρ^0 fibroblasts $\Delta\Psi$ seemed to be even higher than in parental cells, although ATP synthesis was reduced to 60%. In 98% NARP cells, $\Delta\Psi$ was on the same level as in parental cells which is in agreement with [41].

We found that dissipation of mitochondrial membrane potential by CCCP induced mitochondrial fragmentation (Fig. 1). This result agrees with results recently published by others [11,15,42,43] and shows that mitochondrial fusion depends on $\Delta\Psi$. In parental and 60% NARP cells, fragmentation was clearly visible 2 min following CCCP addition but it was not completed as we could see both fragmented and reticular mitochondria in these cells (Fig. 1). At longer times after CCCP addition, all mitochondria appeared fragmented (data not shown). Probably, the fragmented mitochondria observed in ρ^0 could not fuse because their $\Delta\Psi$ was not high enough or structure impaired [44]. Although in 98% NARP cells, $\Delta\Psi$ was on the same level as in parental cells, mitochondria in the former cell line were fragmented. This suggests that fusion of mitochondria depends not only on $\Delta\Psi$. Meeusen et al. [45] have shown that a few factors can influence mitochondrial fusion. For example, ATP synthase can be involved in modifying mitochondrial cristae morphology [46] and, moreover, Geromel et al. [47] have found that NARP mutation is associated with elevated superoxide production and apoptosis.

4.2. Cytoskeletal proteins may play an important role in maintaining the shape of mitochondria

The cytoplasmic environment and the presence of cytoskeletal proteins may play an important role in maintaining the shape and organization of mitochondria in situ [48]. MTs seem to be the major component of cytoskeletal systems responsible for the distribution of mitochondria in mammalian cells but are not required for mitochondrial fusion and fission [11,15]. We did not observe any differences in MTs organization in the osteosarcoma cell lines, even after treatment with CCCP, oligomycin and thapsigargin. However, we found clear differences in arrangement of actin filaments and vinculin in 98% NARP cybrid and ρ^0 cells in comparison to the parental cell line.

Actin filaments form a complex network providing a structural basis for the interaction between intracellular structures and the PM [49–51]. The actin cytoskeleton comprises a cytoplasmic actin network and membrane-associated F-actin. Vinculin is one of the membrane-associated proteins involved in attaching actin filaments to the PM. Our observations indicate that cytoskeletal reorganization can influence mitochondrial distribution and the architecture of the subsarcolemma membrane compartment and therefore may affect CCE. Few studies involving disruption of actin microfilaments demonstrate that the cytoskeleton is integral to CCE regulation. For example, Rosado et al. [49] found that in human platelets the actin cytoskeleton in the vicinity of the PM plays a key regulatory role in Ca^{2+} entry. Lin et al. [52] showed that disruption of cytoskeletal elements diminished calcium influx in myenteric glia cells.

4.3. Mitochondrial dysfunction and calcium influx

As shown in Fig. 5, addition of thapsigargin to osteosarcoma cell lines produced identical transients in $[Ca^{2+}]_c$. This suggests that in the cells suspended in nominally calcium-free media, calcium loading of the ER reached similar levels. Release of calcium ions from the ER activates CCE. Upon addition of 3 mM $CaCl_2$ to the extracellular medium, we observed significant enhancement of $[Ca^{2+}]_c$ in all cell lines treated with thapsigargin. This corresponds to a massive flow of Ca^{2+} across the PM. Energized mitochondria are essential for the activity of CCE in many cell types, including human fibroblast, Jurkat, Ehrlich ascites tumor and glioma cells [27,30,31]. Mitochondria localized close to the PM accumulate Ca^{2+} and protect CCE against feedback inhibition. Therefore, mitochondrial uncouplers as well as inhibitors of the respiratory chain can significantly influence CCE.

However, the lack of an inhibitory effect of CCCP on Ca^{2+} flux into human osteosarcoma cell lines questions the generality of the above statement. Our finding that Ca^{2+} entry into these cells is independent of the mitochondrial energy state strongly suggests that CCE in these cells does not exhibit feedback inhibition by Ca^{2+}. The insensitivity of PM calcium channels to intracellular Ca^{2+} may be related to a high calcium bone environment and be specific to osteoblast-like cells.

Higher amplitude of Ca^{2+} transients observed in the osteosarcoma cells with collapsed $\Delta\Psi$ due to treatment with CCCP resembles, to some extent, the effects of mitochondrial uncouplers on the calcium entry into electrically excitable cells. In excited neurons, mitochondrial sequestration of the entering Ca^{2+} decreases the amplitude of Ca^{2+} spikes and thereby modulates the intensity of calcium signals. Under normal conditions, gradual release of Ca^{2+} accumulated in the mitochondrial matrix prolongs Ca^{2+} signal after the initial stimulus has been turned off. Reduction of the calcium buffering capacity by mitochondria (e.g. by depolarization of the mitochondrial inner membrane) results in the enhanced amplitude and shortened duration of the cytosolic Ca^{2+} signals [53–55].

Ca^{2+} influx rates in 98% NARP and ρ^0 cells, that are approximately 50% slower than in the parental osteosarcoma cell line, may indicate that proper actin filament organization under the PM is important for CCE. In these cell lines, actin cytoskeleton was disturbed (Fig. 3). However, a controversy still exists regarding the role of cytoskeleton in CCE. There are many evidences that cytoskeleton plays an integral role in transmitting to the PM the information about the state of intracellular calcium stores [49,56–58].

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