Two high conductance channels of the mitochondrial inner membrane are independent of the human mitochondrial genome

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Abstract Patch-clamp techniques were used to characterize the channel activity of mitochondrial inner membranes of two human osteosarcoma cell lines: a mitochondrial genome-deficient (ρ^0) line and its corresponding parental (ρ^+) line. Previously, two high conductance channels, mitochondrial Centum picoSiemen (mCS) and multiple conductance channels (MCC), were detected in murine mitochondria. While MCC was assigned to the protein import in yeast mitochondria, the role of mCS is unknown. This study demonstrates that mCs and MCC activities from mouse mitochondria are indistinguishable from those of human mitochondria. The channel activities and their functional expression levels are not altered in cells lacking mtDNA. Hence, ρ^0 cells may provide a model system for elucidating the role of mitochondrial channels in disease processes and apoptosis.
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Key words: Mitochondrion; Channel; Mitochondrial DNA; Patch-clamp; Multiple conductance channel activity; ρ^0

1. Introduction

Changes in mitochondrial permeability have been implicated in an array of mitochondrial-based diseases and, more recently, apoptosis [6–9]. Typically, previous studies of mitochondrial channels have been limited to either yeast or animal (mouse and rat) tissues. Examination of the role of mitochondrial channels in a variety of maladies will be facilitated by extension of these studies to cultured human cells which can be genetically manipulated. For example, ρ^0 cells completely lack mitochondrial DNA (mtDNA) and are incapable of oxidative phosphorylation [10,11]. However, further examination of these cells is warranted since ρ^0 cells maintain mitochondrial compartments and can undergo apoptosis [10].

Two high conductance channels were previously identified in mitochondrial inner membranes using patch-clamp techniques [1–4]. They are the multiple conductance channel (MCC) and the mitochondrial Centum picoSiemen channel (mCS) which have peak conductance of $\sim$1000 pS and 110 pS, respectively. While MCC has been linked to the protein import apparatus of yeast mitochondria [5], the functional role of mCS is the subject of speculation.

In this study, methodologies to examine the channel activity of mitochondria isolated from cultured human cells were developed. It was determined that mCS and MCC activities were conserved between mouse and human mitochondria. Their activities and functional expression levels were not altered by the absence of mtDNA and its gene products. Therefore, ρ^0 cells may provide a system, uncomplicated by classical metabolic constraints, to examine the role of mitochondrial channels in basic disease processes and apoptosis.

2. Materials and methods

The ρ^+ wild-type osteosarcoma cell line (143B) and its derivative ρ^0 cell line (143B206) that lacks mtDNA were grown close to confluence in Dulbecco's modified Eagle medium with 50 µg/ml uridine, 10% fetal bovine serum, and 5% CO_2 at 37°C according to King and Attardi [11]. Harvested cells (pellet volume 0.3–1.2 ml; $1-5\times10^7$ cells) were suspended in 10 ml 1$\times$isolation medium (230 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA pH 7.4) and homogenized with five strokes in a Dounce homogenizer using a drill. The homogenate was centrifuged at $163\times g$ for 80 s (1000 rpm in a Sorvall HB-4 rotor). The supernate containing mitochondria was layered on 5 ml 2$\times$medium (460 mM mannitol, 140 mM sucrose, 10 mM HEPES pH 7.4) and centrifuged at $1020\times g$ (2500 rpm). Mitochondria were pelleted from the interface and top of the gradient by centrifugation for 5 min at $4000\times g$ (5000 rpm). Mitoplasts (mitochondria treated to shear off the outer membrane) were prepared by french pressing the isolated mitochondria in 2$\times$medium at 2000 psi according to the method of Decker and Greenwalt [12]. Mitoplasts were pelleted by centrifugation for 10 min at $4810\times g$ (5500 rpm) and suspended in $\sim$ 300 µl of 1$\times$isolation medium. Mitoplasts from mouse kidney were prepared according to the method of Campo et al. [13].

The yield of mitochondria isolated from various cell lines depended on the extent of breakage of the plasma membranes. The protocols developed for the several cell lines (including human fibroblasts, osteosarcoma, epithelial and HeLa cells, and mouse embryonic stem cells) varied significantly with respect to the number of strokes used for homogenization, and whether the pestle was hand-held or motor-driven. For example, fibroblasts required 10 strokes of the homogenizer with a drill while epithelial cells needed three strokes by hand for best mitochondrial yield.

Patch medium for the experiments with human mitochondria was 150 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.23 mM $CaCl_2$ (10^{-8} M free Ca^{2+}), 5 mM succinic acid, 5 mM $MgCl_2$, 0.2 mM KH_2PO_4 pH 7.4. The medium for the experiments with mouse mitochondria was 150 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.25 mM $CaCl_2$ (10^{-7} free Ca^{2+}) pH 7.4. Voltage-clamp experiments were performed with the inside-out configuration of the patch-clamp technique [14]. The conditions and procedures were the same as previously reported [3,9]. Voltage-clamp conditions were maintained with a Dagan 3900A patch-clamp amplifier in the inside-out mode and voltages were reported as bath (i.e. matrix) potentials. Analysis of current signals by the PAT program was bandwidth-limited to 2 kHz with a low pass filter (model 902 Frequency Devices) and sampling of 5 kHz. Open probability (P_o) was calculated as the percent of the total time spent in the fully open state from amplitude histograms of current traces of 20–60 s duration. Gating charge was calculated from the slope of plots of ln $(P_o/(1-P_o))$ as a function of voltage using the method of

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Abbreviations: MCC, multiple conductance channel activity; mCS, mitochondrial Centum picoSiemen channel; VDAC, voltage-dependent anion-selective channel

Moczydlowski [15]. Data are mean ± standard error for a minimum of four independent determinations.

Oxygen consumption was monitored polarographically with a Clarke oxygen electrode (Yellow Springs Instruments model 53 oxygen monitor) at 37°C. Respiration of cells in growth medium was followed for 5 min prior to the addition of antimycin A. Protein analysis was determined by the bicinchoninic acid assay [16].

Mitochondrial proteins were separated by SDS-PAGE and transferred to nitrocellulose filters for immunoblotting [17]. Filters were decorated with antibodies against cytochrome *c* (Research Diagnostics, Flanders, NJ) and visualized with an AuroProbe BLplus secondary antibody reaction (Amersham, Amersham, UK) [18].

3. Results and discussion

ρ^0 cells have no mtDNA and do not generate ATP by oxidative phosphorylation. While cytochrome *c*, which is nuclear DNA-encoded, is present in mitochondria isolated from ρ^0 cells (data not shown), the respiratory complexes are incomplete. These complexes are not assembled since the mtDNA-encoded subunits are absent [19]. The difference in genotypes of the parental ρ^+ and mtDNA-depleted ρ^0 cells was verified by measuring O_2 consumption. The respiratory rate of ρ^+ cells was reduced from 0.020 to 0.002 μA O/min/mg protein upon the addition of antimycin A, an inhibitor of mitochondrial respiration. In contrast, the respiration of the ρ^0 cells (0.003 μA O/min/mg protein) was insensitive to antimycin A. These data confirm previous findings [10,11] that indicate mitochondrial respiration is absent in ρ^0 cells. However, mitoplasts isolated from ρ^0 cells are indistinguishable from those of the parental cell line when viewed with phase contrast microscopy (Fig. 1).

The activity of MCC of mitoplasts from mouse kidney, ρ^+, and ρ^0 cells was examined with patch-clamp techniques and the results are summarized in Table 1. Current traces show that the MCC from the three types of mitoplasts had the same transition size and voltage dependence (Fig. 2). The P_o was consistently higher with matrix negative potentials. MCC was slightly cationic with a permeability ratio of K/Cl (P_K/P_{Cl}) of 3–7 and no differences in subconductance levels (~ 750, 500, and 320 pS) were detected. Furthermore, the frequency of detecting MCC was the same for mitoplasts from the two human cell lines, and slightly lower for mitoplasts of native mouse kidney.

Current traces recorded from mitoplasts of mouse kidney, ρ^+, and ρ^0 cells showed that mCS activities were indistinguishable (Fig. 3) and these findings are summarized in Table 2. mCS from the three sources had the same conductance, selectivity and voltage dependence. The slope of ln P_o/ $(1-P_o)$ and voltage plots is proportional to gating charge, the net number of charges that move across the membrane
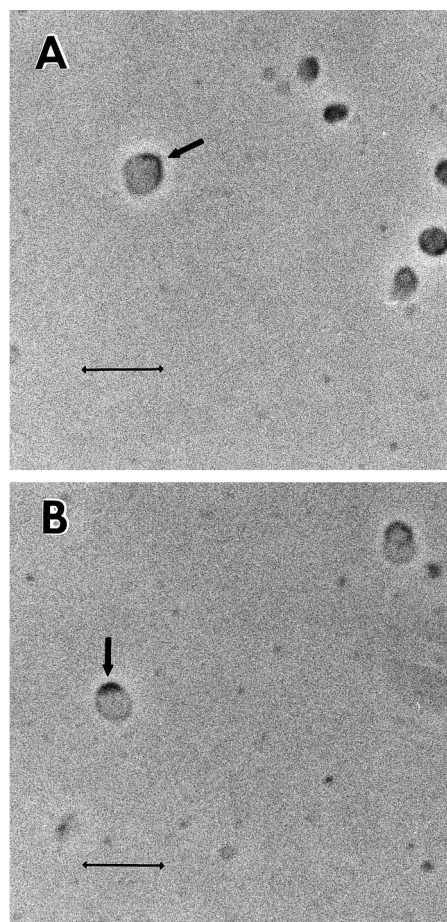

Fig. 1. Mitoplasts isolated from the ρ^+ and ρ^0 human cell lines have the same morphology. Photomicrographs of mitoplasts prepared from ρ^+ (A) and ρ^0 (B) cells were visualized with phase contrast microscopy. Note that mitoplasts from both cell types exhibit the same range of sizes and have the cap regions ($\rightarrow$) associated with mitoplasts [1]. Bar corresponds to 5 μm.

when the channel closes. The voltage dependence of mCS was independent of cell type since data from all three sources were fit with a single line that had a correlation coefficient of 0.99 and a gating charge of 3.4 ± 0.15 (Fig. 4). The frequency of detecting mCS in mitochondria of the two human cell lines was about the same, but this frequency was somewhat lower than in mitoplasts from mouse kidney.

Communication between mitochondria and the rest of the cell is essential to organelle biogenesis and maintenance. Changes in transcription of some genes occur in response to a lack of mitochondrial respiration [19,20]. For example, transcription of cytochrome *c* oxidase subunit IV and adenine

Table 1
A comparison of MCC activity from murine, ρ^+ and ρ^0 mitochondria

	Murine	Human ρ^+	Human ρ^0
Conductance (pS)	1123 ± 78	1020 ± 36	1132 ± 100
Voltage highest P_o	negative	negative	negative
E_{rev} (mV)[a]	23	23 ± 6.2	23 ± 6.2
P_K/P_{Cl}[a]	3–7	3–7	3–7
Subconductance levels	yes	yes	yes
Detection frequency	36%	53%	52%
Number of patches	70	55	42

[a]30:150 mM KCl gradient; mean ± standard deviation, murine values from Kinnally et al. [2].

Table 2
A comparison of mCS activity from murine, ρ^+ and ρ^0 mitochondria

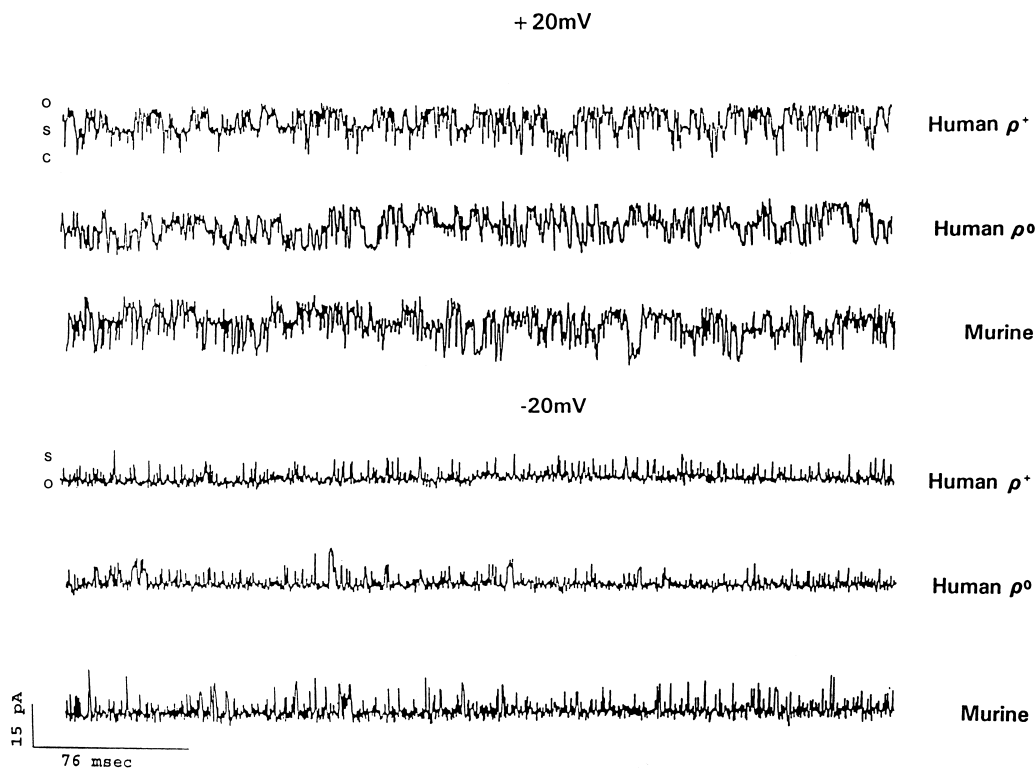|	Murine	Human ρ^+	Human ρ^0
Conductance (pS)	114 ± 2	109 ± 1	107 ± 0.5
Gating charge	3.5 ± 0.1	3.7 ± 0.3	3.2 ± 0.1
Mean open time (ms)	5.0 ± 0.1	4.9 ± 0.1	4.1 ± 0.1
+30 mV (events)	(3465)	(3444)	(4114)
Selectivity	sl. anionic	sl. anionic	sl. anionic
Subconductance levels	yes	yes	yes
Detection frequency	27%	14%	10%
Number of patches	70	55	42

+20mV

Fig. 2. MCC is present in mitochondria from ρ^0 cells. Current traces show MCC activity in patches excised from mitoplasts isolated from mouse kidney, human ρ^+ and ρ^0 cell lines at 20 and −20 mV. Filtration rate was 2 kHz. O is the fully open state, S is the substate, and C is the closed state.
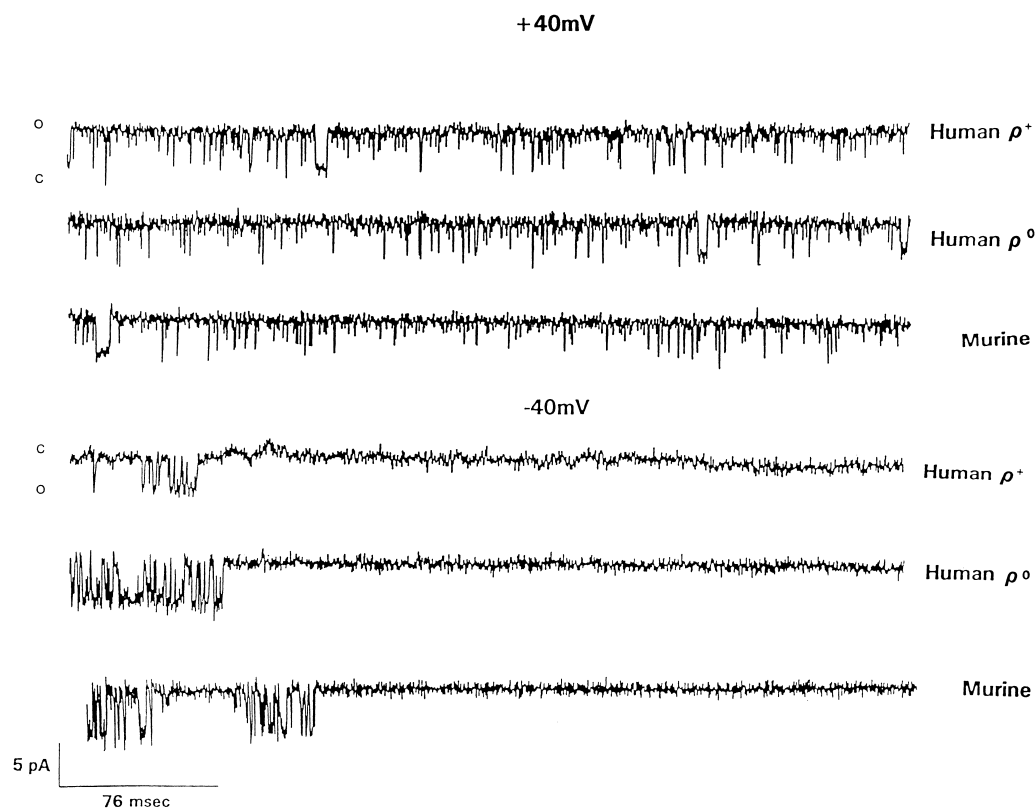
+40mV

Fig. 3. mCS is present in mitochondria from ρ^0 cells. Current traces reveal mCS activity in patches excised from mitoplasts from mouse kidney, human ρ^+ and ρ^0 cell lines at 40 and −40 mV. Other conditions as in Fig. 2.
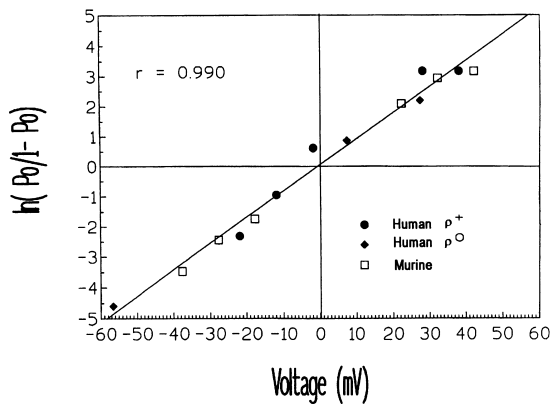
Fig. 4. Voltage dependence of mCS from murine, human ρ^0 and ρ^+ mitochondria is identical. Open probability was determined from total amplitude histograms compiled from 30–60 s of current traces at various voltages (not shown). The data from the three cell types were best fit to a single line with a correlation coefficient (r) of 0.99. The gating charge corresponded to 3.4 ± 0.15.

nucleotide translocator isoforms ANT2 and ANT3 were increased in cultured ρ^0 osteosarcoma cells compared to the parental cell line. Nijtmans et al. found that nuclear-encoded cytochrome c oxidase subunits were translated, imported into mitochondria and processed in cells whose mitochondrial protein synthesis was blocked [19]. However, cytochrome c oxidase was not assembled due to the lack of the mitochondrial-encoded subunits. Interestingly, cytochrome c was detected by immunoblots of mitochondria isolated from the ρ^+ and ρ^0 cells (data not shown). Importantly, the characteristics and frequency of observing mCS and MCC were unchanged by the absence of mtDNA in the ρ^0 cells. These findings eliminate the possibility that mtDNA-encoded proteins or a competent respiratory chain are required for assembly or activity of the two channels.

While MCC is conserved between mammals and yeast [21] and mCS has been detected only in mammalian mitochondria, the proteins responsible for these two channels activities are not known. MCC has been linked to TIM23, a receptor of the protein import complex of the mitochondrial inner membrane of yeast [5]. The availability of knockout strains of yeast has eliminated the possibility that MCC requires VDAC, ANT or the phosphate translocator [5,21,22]. Furthermore, MCC is not affected by growth of yeast under anaerobic conditions [21]. The studies presented here have eliminated the fully assembled ATP synthase [22], cytochrome c oxidase [19], and the membrane-bound portion of NADH dehydrogenase [23] as essential components of MCC and mCS channels. Similarly, cytochrome b has also been dismissed as a candidate protein for mCS and MCC as it is absent in cells lacking mtDNA. Since mCS activity has not yet been observed in yeast, further studies must be done in mammalian systems such as the ρ^0 cells used in this study.

MCC and mCS of human osteosarcoma cells exhibit behav-

iors which are indistinguishable from those of mouse kidney mitochondria. These results have been confirmed with mitochondria from cultured human fibroblasts and breast epithelial cells (unpublished data of Murphy, Schneider, King and Kinnally). The physiological competence of the mitochondria does not appear to affect synthesis or insertion of MCC and mCS channels. Finally, the presence of both inner membrane channels, as well as cytochrome c, in ρ^0 cells makes this simplified system an attractive one for future studies of apoptosis and some mitochondrial-based diseases.

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