The role of mitochondrial dysfunction in regulation of store-operated calcium channels in glioma C6 and human fibroblast cells

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Abstract The store-operated calcium influx into electrically non-excitable cells is greatly modified under the condition of deenergized mitochondria in situ. The rate of calcium influx into cells with empty intracellular calcium stores is greatly diminished when cells were pretreated with 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (a mitochondrial uncoupler) or with 4 μM myxothiazol (an inhibitor of the respiratory chain). We demonstrate that this general phenomenon takes place in the case of transformed (glioma C6 and Ehrlich ascites tumor cells) as well as non-transformed (human fibroblasts) cells. We also demonstrate that the deenergization of mitochondria affects the cellular calcium influx rate and not the calcium pump on the plasma membrane.

Key words: Store-operated calcium channel; Calcium influx; Uncoupler

1. Introduction

Intracellular calcium ions play an important role in the regulation of cell functions (for recent reviews see: [1,2]). The intracellular compartments in which calcium content can vary under physiological conditions include: the cytosol, the nucleus, mitochondria and the endoplasmic reticulum. In all these compartments, changes in $[Ca^{2+}]$ invoke significant modifications in cellular metabolism and function. However, it is the cytosol that physically connects all other intracellular compartments and, thus, is central for calcium metabolism of the cell. Therefore, the Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_c$) is especially tightly controlled.

$[Ca^{2+}]_c$ is influenced by fluxes of Ca^{2+} across various cellular membranes but the ER membrane and the plasma membrane are of particular importance in this respect. In non-excitable cells, two Ca^{2+}-ATPases, one in the ER membrane and the other in the plasma membrane, pump calcium ions into the lumen of ER and out of the cell, respectively. These enzymes are important for maintaining $[Ca^{2+}]_c$ below a micromolar level (typically $[Ca^{2+}]_c$ is between 50 nM and 1 μM) and in a resting cell they counterbalance calcium leaks from the compartments of relatively high $[Ca^{2+}]$, i.e. extracellular fluid (where $[Ca^{2+}]$ usually amounts to 3 mM) and ER lumen (where $[Ca^{2+}]$ is approximately 500 μM [3]). On the other hand, in activated cells an elevation of $[Ca^{2+}]_c$ is produced by an inflow of Ca^{2+} through two specific types of channels, in the ER and in the plasma membrane. The former channels are IP3-sensitive and the latter are so called store-operated calcium channels (SOCs). SOCs are activated when calcium content in the ER is partially or completely reduced. The activities of the channels must be tightly controlled and synchronized to produce $[Ca^{2+}]_c$ changes maximally reaching the level of a few μM. $[Ca^{2+}]_c$ higher than a few μM is generally considered to be harmful for the cell [4].

It has been recently shown that, apart from the ER and the plasma membrane, mitochondria can play an important role in the regulation of SOCs [5–8]. This phenomenon may carry significant physiological consequences and may be a part of the protective mechanism against calcium overloading that may accompany ischemic episodes [8]. These experiment were carried out with human lymphoid Jurkat cells [6,8] and rat hepatocytes [7]. In these cells deenergization of mitochondria greatly impaired the store-operated calcium influx.

Here we study whether these findings are of more general character and can be extrapolated to other types of electrically non-excitable cells. We show that in human fibroblasts and in rat glioma cells the identical phenomenon can be observed. This result means that the participation of mitochondria in the regulation of calcium fluxes across the plasma membrane is rather a general phenomenon. Moreover, we demonstrate that indeed small changes in $[Ca^{2+}]_c$ produced by the addition of Ca^{2+} to the external medium of cells with deenergized mitochondria and empty intracellular calcium stores correspond to the diminution of calcium influx rate and not to the elevation of calcium pumping rate across the plasma membrane.

2. Materials and methods

2.1. Materials

Fura-2/AM and JC-1 ((5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanide iodide) were from Molecular Probes (Eugene, OR, USA). Thapsigargin and ionomycin were purchased from Calbiochem (La Jolla, CA, USA) and myxothiazol was from Boehringer Mannheim (Mannheim, Germany). Other chemicals were from Sigma Chemicals, Co. (St. Louis, MO, USA). The standard incubation medium consisted of 132 mM NaCl, 5 mM KCl, 25 mM HEPES–Na, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 1 mM pyruvate, 5 mM glucose; pH 7.2. Where indicated, 0.12 μM oligomycin, 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 4 μM myxothiazol and 1 μM thapsigargin were added. CCCP, oligomycin and myxothiazol were suspended in dimethylsulfoxide (DMSO).

2.2. Cell culture

C6 glioma cells were grown on glass cover slips in DMEM medium supplemented with 2 mM glutamine (Gibco BRL), 10% fetal bovine

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Abbreviations: $[Ca^{2+}]_c$, cytosolic calcium concentration; SOCs, store-operated calcium channels; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMSO, dimethylsulfoxide

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serum (Gibco BRL), penicillin (100 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere containing 5% CO_2/95% air at 37°C. Human skin fibroblasts originated from healthy individuals. They were grown on glass coverslips in DMEM medium supplemented with 15% (vol./vol.) fetal bovine serum and 1% (vol./vol.) antibiotic–antimycotic solution (Sigma) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

2.3. $[Ca^{2+}]_c$ measurement

Cytosolic free Ca^{2+} was measured with the fluorescent probe, Fura-2. Cells were loaded during the incubation in the culture medium supplemented with 1 μM Fura-2/AM at 37°C for 20 min. After washing out the excess of the probe, Fura2-loaded cells on a cover slip were placed vertically and rectangularly in a cuvette filled with the standard medium. Fluorescence was measured at 30°C in a Shimadzu RF5000 spectrofluorometer set in a ratio mode using 340/380 nm as excitation wavelengths and 510 nm as the emission wavelength. The time resolution of measurements was 1 s. $[Ca^{2+}]_c$ was calibrated for cells in each run using 3 mM externally added $CaCl_2$ and 3 μM ionomycin or digitonin (0.003% final concentration). R_{max} varied from 4 to 6 and from 3 to 4 for glioma cells and fibroblasts, respectively. R_{min} was routinely found to be 0.9.

3. Results and discussion

The plasma membrane of glioma C6 cells becomes permeable towards calcium ions when intracellular calcium stores are partially or fully depleted [9]. This is well illustrated by the control tracing shown in Fig. 1A. Thapsigargin, a specific and potent inhibitor of Ca^{2+}-ATPase in the ER membrane [10], when added to C6 cells immersed in a calcium free medium produces a release of calcium ions from the ER. The initial increase in $[Ca^{2+}]_c$ induced by thapsigargin is interpreted as a sign of this release, and the subsequent return to the basal level of $[Ca^{2+}]_c$ is thought to correspond to the activity of calcium pumps in the plasma membrane and the efflux of calcium ions from the cytosol to the extracellular medium. When the extracellular medium of thapsigargin-treated cells is supplemented with 2.5 mM $CaCl_2$, a significant and rapid elevation of $[Ca^{2+}]_c$ is observed. This is, most probably, a consequence of calcium influx through activated SOCs.

In this elevation of $[Ca^{2+}]_c$ an overshooting is usually observed (Fig. 1A). It may be related to a delayed activation of the plasma membrane calcium-pump [11]. When prior to the addition of thapsigargin, the cells were treated with 0.12 μM oligomycin no significant changes were introduced into this picture (Fig. 1B). However, the picture was drastically different when the cells were initially pretreated with 0.12 μM oligomycin plus 2 μM CCCP (Fig. 1C) or 0.12 μM oligomycin plus 4 μM myxothiazol (Fig. 1D). The addition of oligomycin protects the cells with deenergized mitochondrial membrane against the drainage of glycolytically-generated phosphorylation potential through F_1F_o-ATPase. Therefore, the effect of combined action of CCCP and oligomycin was not related to the decrease in the cellular ATP level, since the equivalent ATP level could be reached from the cytosolic metabolism of glucose. Glioma C6 cells, as all transformed cells, are primarily glycolytic and can fulfill their energy demands exclusively from anaerobic glycolysis [12]. The addition of 2.5 mM $CaCl_2$ to the medium in which the cells, pretreated with the reagents mentioned above and thapsigargin, were immersed produced much smaller elevation of $[Ca^{2+}]_c$ as compared to the control situation (Fig. 1C,D vs. 1A,B). Similar results as those shown in Fig. 1 were obtained with Ehrlich ascites tumor cells (not shown).

CCCP and myxothiazol are very different in their chemical

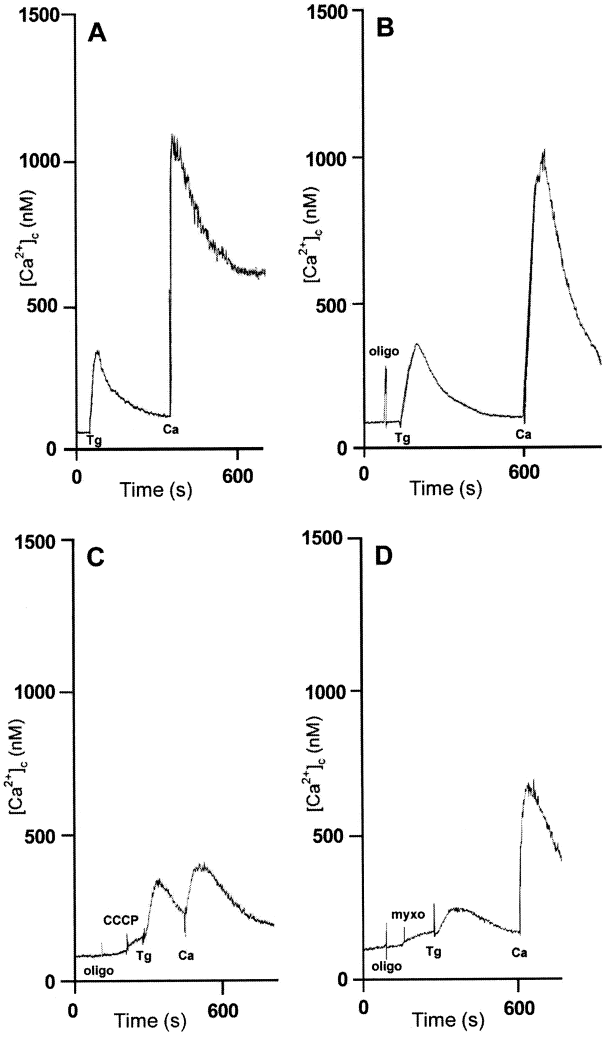

Fig. 1. $[Ca^{2+}]_c$ changes in C6 glioma cells. C6 cells were loaded with Fura2 and immersed in a standard medium. Where indicated, 1 μM thapsigargin (Tg) and 2.5 mM $CaCl_2$ (Ca) were added. A: A control experiment is shown. B: Cells were pretreated with 0.12 μM oligomycin (oligo). C: Cells were pretreated with 0.12 μM oligomycin (oligo) and 2 μM CCCP. D: Cells were pretreated with 0.12 μM oligomycin (oligo) and 4 μM myxothiazol (myxo). (A,B,C,D) One trace, typical of six, six, five and six traces, respectively, is shown.

structure. What they have in common is that they discharge the electrochemical proton gradient ($\Delta\mu_{H+}$) on the inner mitochondrial membrane, CCCP by making the inner membrane permeable to protons and myxothiazol, a potent inhibitor of the b-c_1 segment of the respiratory chain [13], by blocking the generation of $\Delta\mu_{H+}$ by the respiratory chain.

Thus, we conclude that the result previously observed with Jurkat cells [8] concerning the necessity of coupled mitochondria in situ for a pronounced activation of SOCs could be also extrapolated to other lines of transformed cells, represented here by glioma C6 and Ehrlich ascites tumor cells.

It is generally recognized that all transformed cells have some distinct metabolic features when compared to non-transformed cell lines, e.g. their respiration is significantly reduced by glucose addition. This phenomenon, called Crabtree effect from the name of its discoverer, results according to recent hypothesis [12] from some peculiarities in calcium sensitivity

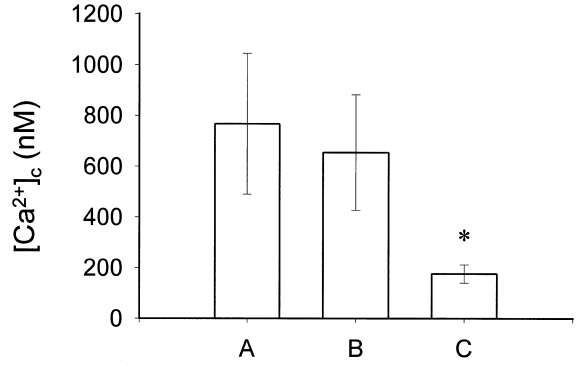

Fig. 2. $[Ca^{2+}]_c$ changes in human fibroblasts. The cells were grown on coverslips and loaded with Fura-2. In column A a control experiment is shown. In column B cells were pretreated with 0.12 μM oligomycin and in column C cells were pretreated with 0.12 μM oligomycin (Olig) and 2 μM CCCP. Column represent mean ± S.D., $^*P < 0.005$ vs. control.

of mitochondrial F_1F_o-ATP [14]. This calcium-related phenomenon is not found in non-transformed cell lines.

In order to establish if the effect of mitochondrial energy status on SOCs can also be found in non-transformed cells, we have carried out experiments with human fibroblasts (Fig. 2). It was found that also in this case the impairment of mitochondrial membrane energy state strongly blocked the activation of SOCs. Therefore, we conclude that the necessity of functional mitochondria for full activation of SOCs holds for various cell types, transformed as well as non-transformed.

It could be argued that the effects of mitochondrial drugs on $[Ca^{2+}]_c$ changes shown in Figs. 1C and 2 are not related to a decrease in the influx through SOCs but rather to a stimulation of calcium efflux through calcium pumps in the plasma membrane. We have attempted to answer this question in experiments with Jurkat cells [8]. However, the results were not clear cut since the responses were rapid and the appropriate rates could not be precisely quantitated. Round shaped Jurkat cells living in suspension are very particular when cell structure is concerned. The central part of the cell is occupied by the nucleus and the rest of the intracellular space is a thin peripheral layer of the cytoplasm. This cell architecture makes $[Ca^{2+}]_c$ responses in Jurkat cells very rapid. Glioma C6 cells and fibroblasts are grown on coverslips and as such are perfect model systems for answering the question regarding whether mitochondrial deenergization influences calcium transports out or into the cell. With cell lines growing on cover slips much of the plasma membrane is excluded from the transport of substances from the extracellular medium into the cytosol and vice versa. Moreover, from the part of the plasma membrane that is available to the transport processes some regions of the cytosol are quite distant. In these cells $[Ca^{2+}]_c$ responses are, therefore, relatively slow and as such suitable for precise quantification in our experimental set-up.

We investigated how the activity of the calcium efflux from glioma cells is influenced by the treatment with oligomycin plus an uncoupler. Simply, we transferred the coverslips covered with glioma cells loaded with calcium (i.e. immersed in the medium supplemented with 2.5 mM $CaCl_2$) into a nominally calcium free medium. The tracings shown in Fig. 3A,B illustrate this experiment. The rates of $[Ca^{2+}]_c$ return to the basal level, when the cells were placed in a nominally calcium free medium, were identical in cells treated and untreated with mitochondrial drugs. Therefore, we conclude that the action of oligomycin plus an uncoupler did not influence calcium pumps in the plasma membrane. This points to the final conclusion that indeed the deenergization of mitochondria in glioma cells primarily influenced the activity of SOCs.

The importance of $[Ca^{2+}]_c$ in the regulation of cellular metabolism is generally acknowledged. The novel role of mitochondria in calcium metabolism, i.e. dependence of SOCs-related calcium influx on the energy status of mitochondria in situ may have profound consequences in cell physiology.

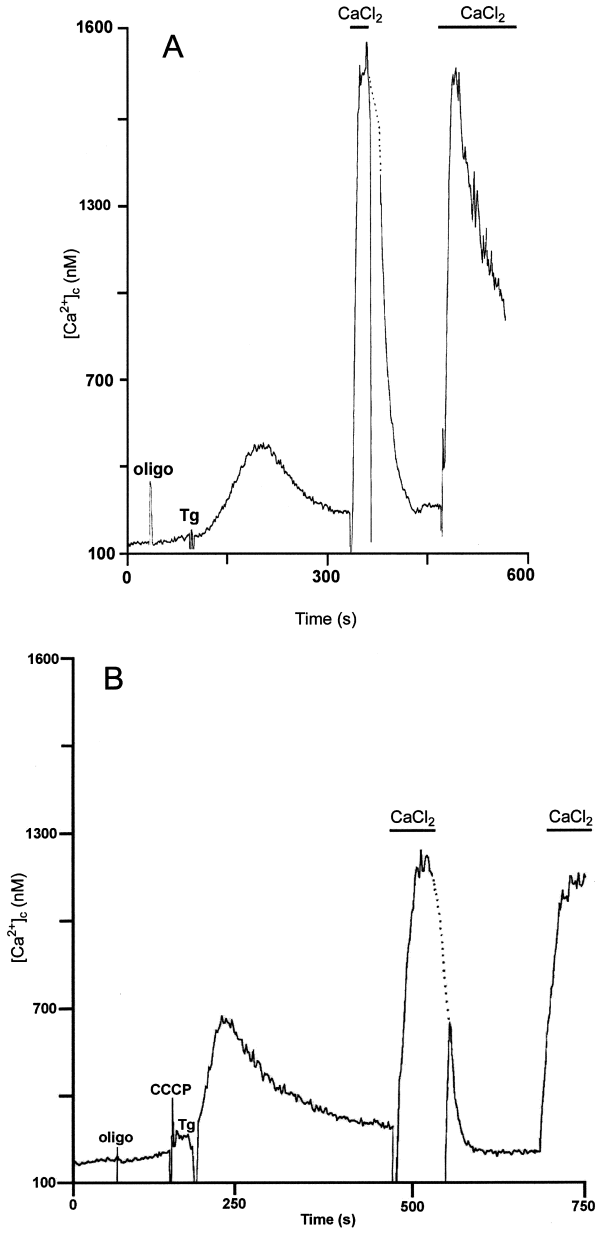

Fig. 3. $[Ca^{2+}]_c$ changes in C6 glioma cells: the effect of $CaCl_2$ withdrawal from the medium. Cells were immersed in a standard medium. Conditions were as in Fig. 1. Where indicated, 1 μM thapsigargin (Tg) and 2.5 mM $CaCl_2$ (Ca) were added. Following a stabilization of $[Ca^{2+}]_c$ on the elevated level, the coverslip was transferred to a cuvette with a nominally calcium free standard medium. A: A control experiment is shown. B: Cells were pretreated with 0.12 μM oligomycin (Olig) and 2 μM CCCP. (A,B) One trace typical of four and five, respectively, is shown.

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