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Irreversibly electropermeabilized yeast retains the capability for ATP synthesis via oxidative phosphorylation

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Abstract

ATP synthesis in irreversibly electropermeabilized yeast *Kluyveromyces lactis* was studied by using different respiratory substrates. The permeabilization itself provoked a dramatic decrease of the total ATP level and the cells lost their ability to synthesize ATP via glycolysis. The addition of exogenous NADH supported ATP synthesis in irreversibly permeabilized cells for up to 4–6 h after substrate addition when the total ATP level became twice that of intact cells incubated for the same period with lactose.

Keywords: Irreversible electropermeabilization; Yeast; ATP synthesis; *Kluyveromyces lactis*

1. Introduction

The application of high intensity electric field pulses on cells provokes a dramatic increase of their plasma membrane permeability, which can be reversible or irreversible in dependence on the electrical parameters [1,2]. The reversible electropermeabilization is now routinely used for electrotransformation, electrofusion, electroloading of macromolecules, substrates, markers and drugs into living cells [3–5].

The irreversible electropermeabilization has gained less attention. In the industry it is applied mostly for food sterilization [6]. During the last years in series of works we demonstrated the possibility for its use as a very efficient new method for enzyme extraction from yeast [7–9]. While characterizing the electroinduced enzyme release we established, that at appropriately selected conditions of electric treatment and postpulse incubation, it is possible to permeabilize irreversibly the plasma membrane of all treated cells, while the integrity of the vacuolar membranes was preserved to a high degree.

In order to better characterize the properties of irreversibly electropermeabilized yeasts, we investigated the possibility to preserve, in such a system, the intactness of another cellular organelle—the mitochondria, by using as a criteria the retained capability of permeabilized cells for ATP synthesis via oxidative phosphorylation.

2. Experimental

2.1. Yeast strain and culture

The experiments were carried out with *Kluyveromyces lactis* strain 2209 haploid, obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), grown on YPL medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) lactose). Growth was checked by turbidimetry (OD_{660}). After reaching the exponential phase of growth, cells were collected by centrifugation at 1500 $\times g$ for 10 min, diluted in deionized water and incubated for 30 min at room temperature. A final centrifugation and a dilution in 0.65 M sorbitol to a final concentration corresponding to 10% wet weight (approximately 1×10^9 cells/ml) then followed.

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2.2. Electropermeabilization

Electric field treatment was performed using an electropulsator (Jouan, France) generating rectangular pulses. Two parallel stainless steel flat electrodes at a distance of 0.25 cm and a length of 0.5 cm were used. They were brought into contact with the bottom of a Petri dish to build an open chamber. Cells were treated with 14 pulses, 2 ms duration, 2 Hz frequency and 4 kV/cm field intensity. Immediately after pulsation 66 μl buffer solution were added to 200 μl cell suspension. The buffer solution was 1.5 M sorbitol, 40 mM potassium phosphate buffer, pH=7, 8 mM $MgCl_2$, 6.8 mM NaCl, 0.4 mM EDTA, 0.4% BSA. The content and the final concentration of incubation medium are as those normally used for determination of ATP synthesizing activity of permeabilized yeast spheroplasts [10].

2.3. Determination of irreversible permeabilization

After pulsation cells were incubated for 2 h at 30 °C. A 5 μl 0.5 mM propidium iodide (PI) were then added to 50 μl cell suspension. The number of fluorescent cells was counted under an inverted fluorescent microscope (HBO Arc lamp, H3 or N2 block filter, Leitz, Germany). The permeabilization was expressed as the percentage of the number of fluorescent cells to the total number of cells.

2.4. ATP determination

The ATP determination is performed by using luciferin–luciferase ATP assay (Roche). Fifteen minutes after electropulsation, 100 μl cell suspension were mixed with 10 μl 50 mM NADH in 0.65 M sorbitol, 0.02 M potassium phosphate buffer, 2.5 mM $MgCl_2$, 2.4 mM NaCl or 10 μl of the same buffer without NADH and incubated for a period from 0.5 to 10 h at room temperature. After a defined period of incubation with substrate, 20 μl of cell suspension (pulsed cells or control) were diluted in 60 μl 15% TCA, containing 8 mM EDTA. Fifty microliters were transferred in 750 μl 100 mM Tris–acetate buffer (pH=7.6, 2 mM EDTA) 1.5 min after dilution. They were kept on ice until ATP determination. A 200 μl 100 mM Tris–acetate buffer (pH=7.6, 2 mM EDTA), 40 μl monitoring reagent (luciferase–luciferine solution), 20 μl assay mixture, 10 μl ATP solution—0.002 μg/ml—were mixed, and the chemiluminescence was determined with LKB-luminometer 1250.

Using this protocol we determined the total ATP content which includes the released ATP and that retained in the cells. The same protocol was applied for ATP determination in intact cells.

2.5. Enzyme activity and protein determination

Protein release determination 4 h after pulsations as well as total protein content in lysate were performed as previously reported [7].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined according Kirshner and Voigt [11] and total protein by using a commercial kit (Bio-Rad, USA) with bovine serum albumin as a standard.

3. Results

3.1. Irreversible permeabilization

Previous studies showed that irreversible permeabilization of and protein leakage from *K. lactis* were under the control of the electric parameters. We selected electrical conditions where about 95% of treated cells were irreversibly permeabilized. The cell envelopes of these cells are permeable for macromolecules. We observed a release of about 40% of total protein as well as 70–80% of GAPDH (150 kDa) activity 4 h after pulsation, the cells being incubated in a buffer (0.1 M phosphate buffer, 0.24 M glycerol, 0.8 mM dithiothreitol, pH=7), used by us in studies on electroinduced enzyme release [8].

In order to avoid the mitochondrial swelling and breakage the pulsation was performed in 0.65 M sorbitol as for permeabilized yeast spheroplasts.

We checked the ATP level in pulsed and control cells without addition of substrate. As shown on Fig. 1A the permeabilization itself led to a considerable decrease of ATP—3–4 times in comparison with intact cells. One should point that in all experiments we determined the total ATP level in the pulsed suspension.

3.2. Oxydative phosphorylation

As a most adequate test for preservation of the mitochondrial intactness we decided to test their functional activity—the ability to synthesize ATP.

The ability for oxidative phosphorylation was investigated by using NADH as respiratory substrate. The choice was driven from the previous knowledge, that this substrate was very efficient regarding the respiratory activity of isolated mitochondria [12]. The yeast mitochondria have a NADH dehydrogenase located on the outer surface of the inner membrane [13] and could use directly as substrate exogenous NADH added in the cytoplasm, as there was no need of translocation of NADH into the mitochondrial matrix.

As shown in Fig. 1A, NADH addition brought an increase in the ATP level, mostly pronounced after 1 h of incubation. After 1.5 h of incubation, the ATP level associated with irreversibly permeabilized cells became similar to that in control ones. NADH supported ATP neosynthesis in irreversibly permeabilized cells continued up to 4–6 h after substrate addition when the total ATP level became twice that of intact cells incubated for the same period with lactose (Fig. 1B).

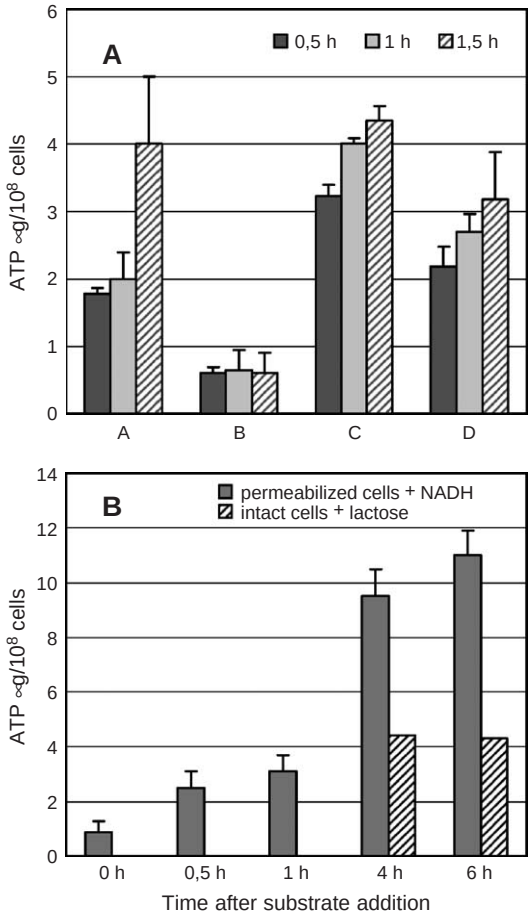

Fig. 1. ATP content of intact and permeabilized cells at different periods after substrate addition. Cells were treated with 14 pulses, 2 ms duration, 2 Hz frequency and 4 kV/cm field intensity. Panel A: A—permeabilized cells with 5 mM NADH, B—permeabilized without NADH, C—intact cells with lactose (2% w/v), D—intact without lactose. Panel B: Kinetics of ATP accumulation in permeabilized cells.

Longer incubation, up to 10 h did not provoke a considerable decrease of the ATP level (data not shown). The electrical conditions we used to permeabilize the cells in this study—14 pulses of 2 ms duration, 2 Hz and field intensity 4 kV/cm were optimal regarding the preservation of ATP synthesis capability in electropermeabilized cells. Application of higher intensities or pulse duration led to decrease of ATP level.

We tested also two other respiratory substrates—ethanol and succinate. Although they were less efficient than NADH (Fig. 2) in both cases we detected ATP level comparable with that of intact cells incubated for the same periods with ethanol as substrate.

3.3. ATP synthesis results from oxidative phosphorylation not from glycolysis

To be able to distinguish the ATP synthesis via oxidative phosphorylation from that by glycolysis, we checked the ATP content after addition of different fermentable substrates—glucose, lactose, trehalose, sucrose to the irreversibly permeabilized cells. The electrical parameters were kept unchanged. No change in the ATP level was observed even after 1 h of incubation, thus leading to the conclusion, that irreversibly permeabilized cells were not able to synthesize ATP via glycolysis.

4. Discussion and conclusions

The results presented here brought direct experimental evidences that one could preserve the mitochondrial intactness in irreversibly electropermeabilized yeast by a proper choice of the electrical parameters and incubation buffer. In fact, irreversibly permeabilized cells are cells, which cannot divide/develop after transfer on nutrient medium. However they retain the capability for ATP synthesis via oxidative phosphorylation for several hours after electrical treatment. This is the first evidence for a retained mitochondrial activity in irreversibly electropermeabilized yeasts.

ATP levels appeared higher in treated cells than in control after several hours of incubation with NADH. We suppose two possible reasons for this—on one hand, the existence in intact cells of a stationary level of ATP which results from the balance of ATP synthesis and consumption reactions, and on the other hand, a very low level of ATP hydrolysis in irreversibly permeabilized cells. The last conclusion is supported by the observation of their steady ATP level up to 10 h.

The lack of glycolysis is probably due to leaks into medium and associated strong dilution of many cofactors, glycolytic intermediates as well as the glycolytic soluble enzymes.

Mitochondria appeared preserved in electrically treated yeasts. This fact could represent an interest from practical point of view. As we showed previously the irreversibly permeabilized yeasts are relatively open systems—cell envelopes being permeable for proteins up to 300 kDa. At suitable electrical conditions their vacuoles remain intact, thus no protease and phosphatase released in the medium.

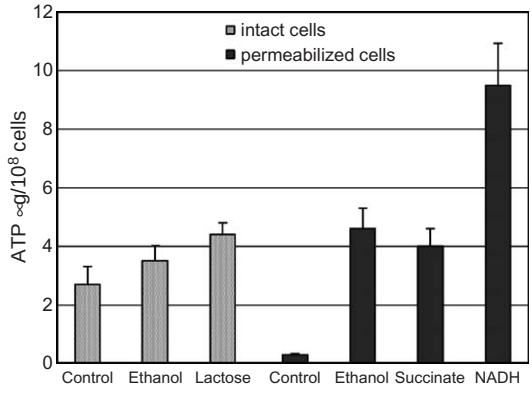

Fig. 2. ATP level in intact and permeabilized cells 4 h after substrate addition. Final substrate concentrations: ethanol—1% (w/v), lactose—2% (w/v), succinate—10 mM, NADH—5 mM.

Furthermore, the electrical treatment did not inactivate the intracellular enzymes, and as shown by the present study the cells retained their capability for efficient ATP synthesis. These particularities of irreversibly electropermeabilized cells, and the fact that they could be obtained easily in large volumes and from any strain, could render them a very suitable system for in situ studies of a number of cellular processes—transcription, translation, intracellular trafficking etc. at conditions close to those of intact cells.

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