Intracellular Delivery of Trehalose into Mammalian Cells by Electropermeabilization

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Abstract. The disaccharide trehalose is increasingly being used as a very efficient stabilizer of cells, membranes and macromolecules during cryo- and lyoconservation. Although extracellular trehalose can reduce cryo- and lyodamage to mammalian cells, the sugar is required on both sides of the plasma membrane for maximum protection efficiency. In the present study, mouse myeloma cells were loaded with the disaccharide by means of reversible electropерmeabilization in isotonic trehalose-substituted medium, which contained 290 mM trehalose as the major solute. By using the membrane-impermeable fluorescent dye propidium iodide as the reporter molecule, optimum electropulsing conditions were found, at which most permeabilized cells survived and recovered (i.e., resealed) their original membrane integrity within a few minutes after electric treatment. Microscopic examination during the resealing phase revealed that electropulsed cells shrank gradually to about 60% of their original volume. The kinetics of the dye uptake and the volumetric response of cells to electropulsing were analyzed using a theoretical model that relates the observed cell volume changes to the solute transport across the transiently permeabilized cell membrane. From the best fit of the model to the experimental data, the intracellular trehalose concentration in electropulsed cells was estimated to be about 100 mM. This loading efficiency compares favorably to other methods currently used for intracellular trehalose delivery. The results presented here point toward application of the electropерmeabilization technique for loading cells with membrane-impermeable bioprotectants, with far-reaching implications for cryo- and lyopreservation of rare and valuable mammalian cells and tissues.

Key words: Electroporation — Disaccharide — Membrane permeability — Electric breakdown — Electroinjection — Volumetry

Introduction

The disaccharide trehalose is found at high concentrations in organisms that are capable of withstanding various environmental stress conditions. Many yeasts, plants and some animals naturally synthesize trehalose, which protects the structural integrity of cells during extreme dehydration and cold (Drennan et al., 1993; de-Araujo, 1996; Crowe & Crowe, 2000). Due to its extraordinary properties, trehalose is increasingly being exploited in biomedicine and biotechnology as a very efficient stabilizer of frozen and dry macromolecules (e.g., antibodies, enzymes, etc.), artificial and natural membranes (Rossi et al., 1997; March & Clark, 2000; Puhlev et al., 2001). Medical applications of trehalose include lyo- and cryopreservation of blood, sperm, tissues and whole organs for storage, transport and surgical transplantation (Beattie et al., 1997; Woelders, Matthijs & Engel, 1997; Eroglu et al., 2000; Guo et al., 2000; Wolkers et al., 2001).

Several molecular mechanisms have been suggested by which trehalose protects cells during freezing and drying. These involve stabilizing effects on both cellular proteins and membranes by formation of hydrogen bonds and/or by water replacement, inhibition of intracellular ice formation, increase of the surface energy between the cell membrane and bulk solvent, etc. (de-Araujo, 1996; Oliver, Crowe & Crowe, 1998; Wolfe & Bryant, 1999; Tsvetkova et al., 1998; Takahashi, 1999, Crowe & Crowe, 2000; Lambruschini et al., 2000). Although extracellular trehalose can reduce cryo- and lyodamage to cells, the

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disaccharide is usually required on both sides of the cell membrane for maximum protection efficiency (Paiva & Panek, 1996; Erglu, Toth & Toner, 2001; Wolkers et al., 2001). Depending on the cell type, 150–200 mM intracellular trehalose is necessary for improved survival of frozen mammalian cells after thawing (Erglu et al., 2000, 2001). In contrast to freeze-thaw protocols, much less intracellular trehalose (20–25 mM) is required for efficient preservation of mammalian cells by freeze-drying (Wolkers et al., 2001).

Various experimental approaches have been developed for the introduction of trehalose into mammalian cells, which can neither synthesize nor actively accumulate this disaccharide. Some of these in vitro techniques make use of the leakiness of cell membranes during the phase transition (Beattie et al., 1997), the fluid-phase endocytosis (Wolkers et al., 2001) or a pore-forming protein inserted into the plasma membrane to introduce exogenous trehalose (Erglu et al., 2000). In a different approach, the genes for trehalose synthesis have been transferred into the target mammalian cells using viral vectors (Guo et al., 2000). Irrespective of the technique, the combination of intra- and extracellular trehalose was found to enhance greatly the survival of mammalian cells in the frozen or dry state.

An alternative approach that might allow to overcome the natural impermeability of cell membranes to trehalose is electropermeabilization (for review see Zimmermann & Neil, 1996). This technique (also known as electroporation or -injection) provides a well-proven tool for the introduction of various membrane-impermeable xenomolecules (such as drugs, hormones, proteins, plasmids, etc.) into living cells as well as the controlled release of intracellular substances. Electropermeabilization is based on the temporary increase of the membrane permeability due to reversible electric breakdown of the plasma membrane upon application of external high-intensity field pulses of very short duration (Zimmermann, Pilwat & Riemann, 1974). This field-pulse technique has gained common acceptance because it is more controllable, reproducible, and efficient than other methods for intracellular delivery of foreign molecules (Friedrich et al., 1998, Spiller et al., 1998; Delteil, Teissié & Rols, 2000).

In the present study, the introduction of trehalose into mouse myeloma cells (Sp2 line) was performed by means of electropermeabilization. Cells were suspended in isotonic trehalose medium (ITM, containing 290 mM trehalose as the major solute) and subjected to mild electropulsing conditions that produced no detectable loss of cell viability. The permeabilized cells recovered (i.e., resealed) within few minutes their original membrane impermeability to trehalose and other small solutes. The resealing time constant was about 4 min at room temperature, as assessed from the kinetics of the uptake of the fluorescent dye propidium iodide (PI). The volumetric response of cells to electropulsing was analyzed using a theoretical model that allowed the quantitative evaluation of the intracellular trehalose delivery.

Using mild field-pulse conditions, about 100 mM trehalose could be introduced into the cytosol of living cells. The loading efficiency obtained here with electroporation compares favorably to those reported for other techniques currently used for intracellular trehalose delivery.

Materials and Methods

Cell Culture

The murine myeloma cell line Sp2/0-Ag14 was cultured in RPMI 1640 complete growth medium (CGM), supplemented with 10% (v/v) fetal calf serum (FCS; PAA, Linz, Austria), at 37°C under 5% CO₂. The cells were kept in the exponential growth phase by subculturing two or three times a week.

Pulsing Medium

Isotonic trehalose medium (ITM) contained 290 mM trehalose (Sigma, Deisenhofen, Germany) and 5 mM KCl as the major osmoticum and electrolyte, respectively. Osmolality and conductivity were determined to be 300 mOsm and 0.9–1.1 mS/cm (at 22°C) by means of a cryoscope (Osmomat 030, Gonotec, Berlin, Germany) and a conductometer (Knick, Berlin, Germany), respectively. pH of the medium was about 7.3. The reasons for using low-conductivity medium are discussed in detail elsewhere (Mussauer et al., 1999; Mussauer, Sukhorukov & Zimmermann, 2001).

Electroporation

Electropermeabilization of Sp2 cells suspended in ITM was performed by means of the Multiporator distributed by Eppendorf (Hamburg, Germany). This instrument generates exponentially decaying field pulses with peak intensities of up to 1.2 kV and decay times between 15 and 500 µsec (Friedrich et al., 1998). Most experiments were performed with the commercial cuvettes purchased from Eppendorf (Hamburg, Germany). These cuvettes, consisting of two planar aluminum electrodes (2 cm² area) spaced by d = 2 mm, were filled with 400 µl of cell suspension. Alternatively, a chamber consisting of two parallel stainless steel wire electrodes with a diameter of 200 µm was used for microscopic observations of cells during and after electropulsing. The electrodes were mounted on a glass substrate at a distance d of 560 µm. Typically, 10–20 µl of cell suspension was pipetted between the wire electrodes and covered with a glass coverslip. For both electroporation chambers, the initial applied field strength E₀ was calculated from the supplied pulse voltage V₀ as E₀ = V₀/d. All electropermeabilization experiments were performed at room temperature (20–22°C).

Microscopy

Observations were made with a microscope (BX 51 Olympus, Hamburg, Germany) using transmitted light to examine the volumetric cell response to electropulsing (see Results and Appendix 2). Microphotographs were taken before and every one minute after