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How transient alterations of organelles in mammalian cells submitted to electric field may explain some aspects of gene electrotransfer process

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ABSTRACT

Electric pulses can be used to transiently permeabilize the cell plasma membrane. This method is nowadays employed as a safe and efficient means to deliver therapeutic molecules into target cells and tissues. Despite the large bulk of literature on this topic, there is a lack of knowledge about the mechanism(s) of molecule delivery. The behavior of the cells both while the field is on and after its application is indeed not well described. Questions about cell organelle alterations remain unanswered. We report here evidence for a number of ultrastructural alterations in mammalian cells exposed to electric pulses. Specifically, CHO cells were subjected to trains of 10 pulses lasting 5 ms using an electric field of 800 V/cm, i.e. under conditions leading both to membrane permeabilization, gene transfer and expression. Cells were observed to undergo morphological alterations of the mitochondria and nucleus. These modifications, detected in the minutes following pulse delivery, were transient. They may have direct consequences on molecule delivery and therefore may explain various aspects of the mechanisms of DNA electrotransfer.

1. Introduction

Electropermeabilization, also named electroporation, is an efficient and elegant way to deliver molecules into cells and tissues. This physical process results from the application of electric pulses that transiently and locally permeabilize the cell plasma membrane. Introduced in the early 70s, it was developed in the 80s for gene delivery [1,2]. During the past four decades, advances in fundamental and experimental electroporation research have allowed the translation of electroporation-based technologies to the clinic [3]. Antitumoral drug delivery to patients and pets, a process called electrochemotherapy [4–7], is nowadays used in an increasing number of countries as a palliative treatment. The transfer of genes into various tissues including the skin, liver, tumors and skeletal muscle, a process called electrogenetherapy [8–13], is under investigation in clinics [14]. A phase I dose escalation trial of plasmid interleukin-12 (IL-12) electroporation carried out in patients with metastatic melanoma, indicated this modality to be safe and efficient [15]. In an attempt to increase systemic antitumor effectiveness of electrochemotherapy, electrotransfer of genes with an immunomodulatory effect can be used as adjuvant treatment [16].

Single-cell studies enabled the description of the mechanisms of molecule electrotransfer. Small molecules have a free access to the cytoplasm of electropermeabilized cells [17,18]. Their transfer into the cytoplasm occurs both during electric pulse application and in the minutes following it, as long as the plasma membrane stays permeable [19]. In contrast, introduction of larger molecules such as plasmid DNA requires a number of consecutive steps: migration of DNA towards the membrane, DNA insertion into the membrane, translocation across the membrane, migration of DNA towards the nucleus and finally transfer of DNA across the nuclear envelope [20–23]. The two first steps occur while the electric field is on, the three following ones occur from very few minutes to several hours following the electric field application. Membrane permeabilization is indeed induced during electric pulse delivery. But cells remain permeable for a while after the field is switched off [14]. The actual permeabilization process and the subsequent behavior of the cells both while the field is on and after its application are not well understood [24]. Transfection appears to be a cell-mediated process where ATP controls cytoplasmic DNA migration towards the nucleus and its expression [25]. Evidence of the participation of the actin cytoskeleton in the first steps of DNA electrotransfer has been reported [26]. Further experiments revealed that DNA is actively transported by the cytoskeleton [27].

Open questions therefore exist about the possible cellular alterations and their consequences on molecule delivery [28]. Indeed, despite the large number of publications on electropermeabilization, there are no studies that really succeed in visualizing the phenomenon at an ultrastructural level. Large “pore-like crater” structures or “volcano funnels” of 50 nm to 0.1 μm diameter, have been observed in red blood cells [29], but can be considered as experimental artifacts, resulting from the enlargement of smaller primary pores by osmotic swelling. No such defects were ever observed under isoosmolar conditions or in other cell models. Electrically induced fusion of human erythrocytes has been studied by rapid quench freeze-fracture electron microscopy. The

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electric pulse causes a brief disruption of the aqueous boundaries which separate the cells. Within 100 ms, the fracture faces exhibit discontinuous areas predominantly free of intramembranous particles. At 10 s, the majority of the discontinuous areas and point defects disappeared, except at sites of cytoplasmic bridge formation [30]. By using scanning electron microscopy performed on CHO cells, Escande-Géraud and collaborators observed numerous microvilli and blebs almost immediately after application of the field. The appearance of osmotic pressure-dependent blebs was indicative of local weakening of the plasma membrane. Most of these effects were fully reversible and disappeared within 30 min [31]. In a more recent work, Spugnini and coworkers described ultrastructural alterations in the cellular membranes following exposure of orthotopic melanomas and red blood cells to electric pulses [32]. The freeze-fracturing analysis of both cell types revealed defects in the dynamic assembly of lipids and proteins, which generate "areas with rough structure" and intensive clustering of intramembrane proteins. Such modifications could be the hallmarks of lipid and protein alterations, of reduced protein cohesion, and of changes in lipid orientation inside cell membranes.

But, in spite of this knowledge, there is still a lack of information on possible direct or indirect effects of electropermeabilization on organelles such as the nucleus and the mitochondria which are involved in gene delivery and expression processes [33,34]. Therefore, the aim of the present study was to make progress in our knowledge of electrotransfer mechanisms and more specifically on the cellular description at the cell organelle level and their possible consequences on molecule transfer. In order to obtain direct insight into cell modifications, we investigated the major ultrastructural alterations occurring after delivery of trains of electric pulses leading to efficient DNA delivery. Cells were chemically fixed from the very few minutes up to 2 h after being pulsed and were observed by transmission electron microscopy. The ultrastructural results are discussed here taking into account current knowledge about the mechanism(s) of cell membrane electropermeabilization and gene delivery, proposing, whenever possible, links with both the existing experimental and the theoretical backgrounds.

2. Materials and methods

2.1. Cells

Chinese hamster ovary (CHO) cells were used. The WTT clone was selected for its ability to grow on Petri dishes or in suspension under gentle agitation in MEM medium as previously described [35,36].

2.2. Electropulsation

Electropulsation was performed by using a CNRS cell electropulsator (Jouan, St Herblain, France), which delivered square-wave electric pulses. An oscilloscope (Enertec, St Etienne, France) monitored pulse shapes. Stainless steel parallel electrodes (diameter 0.5 mm, length 10 mm, inter-electrode distance 7 mm) were connected to the voltage pulse generator [37]. CHO cells were electropulsed in pulsation buffer (10 mM K_2HPO_4/KH_2PO_4, 1 mM MgCl2, 250 mM sucrose, pH 7.4) by application of electric pulses known to induce small molecules as well as macromolecules loading into CHO cells [37].

Two different settings of electric pulses were used: i) 10 pulses lasting 100 μs applied at 1.3 kV/cm and at a 1 Hz frequency that allowed membrane permeabilization of the whole cell population to small molecules while preserving cell viability [36] and ii) 10 pulses lasting 5 ms applied at 800 V/cm at a 1 Hz frequency that allowed DNA plasmid electrotransfer with cell viability up to 75% [38]. In some gene transfer experiments, cells were pulsed in the presence of 23 μg of plasmid DNA labeled with a 10 nm diameter gold particle (Gene Therapy systems).

2.3. Electron microscopy

For the analysis of ultrathin sections, cells were pulsed in 100 μl of pulsation buffer, fixed after a defined lapse of time with 100 μl of a 1.75% glutaraldehyde in 0.05 M cacodylate buffer at room temperature for 10 min and then centrifuged at 2000 *g* in a BEEM capsule (SPI Supplies, West Chester, PA). The supernatant was discarded and replaced by pure fixation buffer for 1 h at room temperature. After post fixation with 2% osmium tetroxide in water (4 °C, 3 h), cells were dehydrated through graded ethanol concentrations with a final epoxy-1,2-propane dehydration. Samples were then embedded in Epon resin (Fluka, Switzerland). Ultrathin sections, obtained with an LKB ultra microtome (LKB, Bromma, Sweden), were stained with uranyl acetate and lead citrate. Finally, ultrathin sections (i.e. 150–200 nm) were examined with a Philips EM 301 transmission electron microscope (Eindhoven, The Netherlands).

3. Results

The aim of our work was to detect cell alterations that may be induced during the electropermeabilization process and that may explain some aspects of molecule delivery. Thus, transmission electron microscopy was used to analyze the effect of electric pulses leading both to permeabilization, efficient gene transfer and expression. Cells were submitted to electric field conditions known from previous studies to lead to efficient plasma membrane permeabilization and gene expression, 90% and 40% respectively, while preserving cell viability up to 75% [38]. As previously described with this cell type, survival is not strongly affected by these electrical parameters [39,40]. Under those conditions, the lifetime of the permeabilized state was 10 min. In a first set of experiments, CHO cells submitted to electric pulses were compared to control cells. Secondly, we designed experiments in the presence of plasmid DNA.

The plasma membrane of control cells did not present any membrane discontinuities. In the cytoplasm, the different organelles (mitochondria, endoplasmic reticulum, nuclei) were easily visualized (Fig. 1A). As expected [41], the mitochondrial matrix of the cells was clearly defined (no difference of contrast between the matrix and cytoplasm could be seen) and the crests were parallel (Fig. 1B). The inside of the reticulum was bright and its flattened cisternae were parallel. The perinuclear chromatin was under a condensed form (Fig. 1A).

Under electric field conditions leading to membrane permeabilization to small size molecules (i.e. 10 pulses lasting 100 μs at a 1.3 kV/cm intensity and 1 Hz frequency), no visible structural organelle alterations were observed (data not shown) in agreement with previous experiments [31]. In contrast, under longer pulse duration (i.e. 10 pulses lasting 5 ms at a 0.8 kV/cm and 1 Hz frequency), conditions leading to gene transfer and expression, transient alterations were detected (Fig. 2). Cells were indeed submitted to electric pulses and then fixed after different time lapses following their delivery. At 1 min, 80% of cells revealed ultrastructural changes (Table 1). The main modifications were found at the level of the intracellular organelles, no evidence for plasma membrane alteration being observed. Large vacuoles were present in the cytoplasm (Fig. 2A). The mitochondrial matrix became dark and mitochondrial crests were dilated (Fig. 2B). The endoplasmic reticulum was also dilated (Fig. 2C) and its inside became darker (Fig. 2D). The nuclear membrane presented breaks (Fig. 2E) and pores were dilated (Fig. 2F). The nucleoli were no longer visible and the perinuclear chromatin was under a decondensed form. Maximum effects were obtained 3 min after pulse delivery as shown in Table 2 that reports a quantitative analysis of the percentages of cells having altered intracellular structures along time. Immediately after the pulses, cells were seen to be affected at the mitochondria, chromatin and nuclear envelope levels. These alterations appeared to be transient. At 10 min, 2 populations of cells were observed: cells which were similar to the control cells (Fig. 3A) and cells