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Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses

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ABSTRACT

High-frequency bipolar electric pulses have been shown to mitigate undesirable muscle contraction during irreversible electroporation (IRE) therapy. Here, we evaluate the potential applicability of such pulses for introducing exogenous molecules into cells, such as in electrochemotherapy (ECT). For this purpose we develop a method for calculating the time course of the effective permeability of an electroporated cell membrane based on real-time imaging of propidium transport into single cells that allows a quantitative comparison between different pulsing schemes. We calculate the effective permeability for several pulsed electric field treatments including trains of 100 μs monopolar pulses, conventionally used in IRE and ECT, and pulse trains containing bursts or evenly-spaced 1 μs bipolar pulses. We show that shorter bipolar pulses induce lower effective membrane permeability than longer monopolar pulses with equivalent treatment times. This lower efficiency can be attributed to incomplete membrane charging. Nevertheless, bipolar pulses could be used for increasing the uptake of small molecules into cells more symmetrically, but at the expense of higher applied voltages. These data indicate that high-frequency bipolar bursts of electrical pulses may be designed to electroporate cells as effectively as and more homogeneously than conventional monopolar pulses.

1. Introduction

Biological membranes are critical to maintaining cellular homeostasis by isolating a cell's interior from its extracellular environment. The cell utilizes its membrane as a barrier to general transport, but allows for the controlled exchange of valuable nutrients, chemical signals, and waste products through transmembrane structures, which shuttle molecules into and out of the cell and maintain the precise homeostatic balance necessary for the cell's function and survival. When biological membranes are exposed to sufficiently intense pulsed electric fields (PEFs), their permeability increases, enhancing the molecular exchange between the cell and its environment. This phenomenon, known as electroporation or electropermeabilization, enables processes like gene transfection [1,2] and chemotherapy [3,4] to be performed much more efficiently. Molecular dynamics simulations have suggested that the formation of pores in the lipid bilayer occurs when water molecules align at the water-bilayer interface and are driven through the hydrophobic bilayer core by local electric field gradients [5–8]. When water molecules cross the membrane, the surrounding lipid head groups usually follow the penetrating water molecules into the pore to energetically stabilize the pore structure [6,9,10]. This restructuring of the lipid bilayer has been hypothesized to alleviate the electrotension caused by collection of oppositely-charged ions on each side of the membrane [11,12]. As long as the electric field is sustained, the pore can further expand in size facilitating the transport of ionic/molecular species across the pore [13–15]. Nevertheless, other mechanisms have been proposed to explain the increased cell membrane permeability caused by electric pulses, such as lipid peroxidation and restructuring of the membrane due to changes in membrane protein conformation [16,17].

Electroporation has been used clinically to either directly ablate tumor tissue or transiently increase membrane permeability to enhance drug delivery at the target sites inside the cell interior. In irreversible electroporation (IRE), the cellular membrane is disrupted to generate an irrecoverable homeostatic imbalance [18–21]. In gene electrotransfer (GET) [22,23] or electrochemotherapy (ECT) [24–26], electroporation enables therapeutic molecules to be more efficiently delivered into cells. During an ECT procedure, a drug such as cisplatin [27,28] or bleomycin [29–31] is first injected into a tumor site and is shortly followed by a PEF treatment, enabling the chemotherapeutic drugs

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to be administered with greater potency by overcoming the cell membrane's transport barrier. Because ECT is delivered locally to the tumor site, minimal systemic side-effects present as a direct result of the treatment while retaining an equivalent or greater clinical efficacy compared to traditional chemotherapy [32–35]. GET is a non-viral gene transfer method that depends on PEF treatment to enhance the delivery of therapeutic genetic material [36,37]. By inserting DNA carrying specific genetic code into cells, GET enables targeted introduction, replacement, or inactivation of selected genes. PEF treatment has dramatically improved gene-transfer efficiencies in tissues such as liver, skin, and skeletal muscle [22,38–40].

IRE technology has been used to treat tumors in canine brain tissue [41], human and porcine liver tissue [42–45], and human and porcine pancreatic tissue [46–48]. By destroying malignant cells while mitigating damage to critical stromal tissue components [49,50], it enables the treatment of tissues around critical structures that would otherwise render the site untreatable. A notable recent improvement in IRE has been termed high-frequency IRE (HF-IRE) and replaces the long monopolar pulsing schemes traditionally used in IRE (80×100 μs-long pulses delivered at 1 Hz) with bursts of short bipolar pulses [20]. These bursts of short pulses partially mitigate intra-operative impedance changes [51] and virtually eliminate muscle contractions [20,52–54] during the treatment to potentially improve both current treatment planning algorithms [20,55] and the procedural safety for the patient due to the reduced need for neuroparalytic drugs typically required to inhibit muscle contraction. For the same reason, bursts of short pulses could also be advantageous in ECT and GET, which have historically utilized pulse widths of hundreds of microseconds to milliseconds to permeabilize the cell membrane.

In the food processing industry, however, PEFs comprised of trains of pulses of 1–10 μs are routinely used to kill pathogenic and spoilage microorganisms around vegetal and animal tissue [56,57]. Only recently has such electroporation been studied in mammalian tissue for medical applications that utilize controlled, square electrical pulses on the order of one microsecond [19,58]. The observations about PEFs with microsecond pulse widths from the field of PEF-based food processing do not directly provide information on the efficiency of these pulses to enhance molecular transport across mammalian cell membranes. Consequently, more detailed study of the molecular transport occurring between a mammalian cell and its environment is needed during these types of pulses. We thereby aimed in the present study to compare membrane permeabilization obtained with 1 μs bipolar pulses and conventional 100 μs pulses, used in IRE and ECT.

In order to characterize molecular transport induced by different pulsing protocols, we performed real-time microscopic imaging of propidium iodide (PI) transport at the single cell level during and after PEF treatment. The measured changes in PI fluorescence intensity due to the uptake and subsequent binding of PI ions to intracellular nucleic acids enabled us to calculate the time course of the effective permeability of a cell membrane. This method can be further refined and validated against mechanistic models of the electroporation and membrane permeabilization processes to advance electroporation-based treatments and therapies. The results presented herein demonstrate that high-frequency bipolar electrical pulses may be designed to achieve similar degrees of electroporation as current IRE, ECT and GET pulsing schemes, but induce more symmetrical transmembrane uptake of small molecules than conventional treatments.

2. Materials & methods

2.1. Cell preparation

Chinese hamster ovary (CHO-K1) cells were obtained from the European Collection of Authenticated Cell Cultures and grown in HAM-F12 medium (PAA, Austria) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany), 1 mM L-glutamine (StemCell Technologies, Vancouver, Canada), 5 mg/mL gentamicin (Sigma-Aldrich), and 0.01 μL/mL penicillin-streptomycin (PAA), at 37 °C under 5% CO_2. Cells were allowed to become 70–80% confluent before being trypsinized, resuspended, and transferred into glass-bottom Lab-Tek II chambers (Nalge Nunc, Wiesbaden, Germany) (7×10^4 cells in 1 mL of growth medium) to easily observe the cells during the experiment. Cells were then incubated at 37°C under 5% CO_2 for 2 h during which they adhered to the bottom of the chamber but retained roughly spherical shape. After 2 h, the growth medium was removed and replaced with low-conductivity, isoosmotic (292 mOsm/kg) potassium phosphate electroporation buffer (KPB: 10 mM KH_2PO_4/K_2HPO_4 in a ratio of 40.5:9.5, 1 mM $MgCl_2$, and 250 mM sucrose; pH = 7.2; electrical conductivity of 0.16 S/m) at 25 °C containing 0.15 mg/mL propidium iodide (PI) (Life Technologies, Carlsbad, USA). As KPB is approximately 10-fold less conductive than the growth medium, it was used to reduce the electric current and minimize the effects of Joule heating on the cells during PEF treatment.

2.2. Electrode design, numerical modeling of electric field distribution, and thermal considerations

Two parallel Pt/Ir alloy (90:10) wire electrodes (0.8 mm diameter wires spaced 4 mm edge-to-edge) were inserted into the Lab-Tek II chamber seeded with cells (as described) on the bottom of the glass surface. With the goal of tightly controlling the electric field to which the cells were exposed, the electric field distribution between the electrodes was simulated numerically in COMSOL Multiphysics (Version 5.1, Comsol, Burlington, MA) using the electrostatics module. The numerical calculations showed that the cells centered between the electrodes (which were monitored in the experiments) experience practically homogeneous electric field. The electric field was simulated using a 1 V test pulse (2.2 V/cm along the midline between the electrodes) to give the normalized distribution of the electric field as a result of the applied potential (Fig. 1, center and right panels).

The energy delivered during electroporation treatment was approximated in a manner similar to [59], assuming the load may be modeled as having parallel resistive and capacitive components, $R(t)$ and $C(t)$, leading to resistive current $I_R(t)$ and capacitive current $I_C(t)$, respectively. Assuming that the resistance of the medium is constant $R(t) = R$ and ideal pulses are delivered (*i.e.* each pulse starts and ends as $V_0 = V_f = 0$), the capacitive current may be neglected, giving

$$E = \int_0^{\tau_{on}} V(t)(I_R(t) + I_C(t))dt = \int_0^{\tau_{on}} \left(\frac{V^2(t)}{R(t)} + V(t)C(t)\frac{dV}{dt}\right)dt \approx \sum_{i=1}^{N} \frac{V_i^2}{R}\tau_i = \frac{V^2}{R}\tau_{on}, \quad (1)$$

where $\tau_{on} = \sum \tau_i$ is the total energized time of the electrodes during the treatment scheme, N is the total number of pulses in either polarity, τ_i is the pulse width of each pulse, and V_{app} is the steady-state amplitude of the pulse. Each of the treatments was designed to have equivalent τ_{on} and the applied potentials $|V_{app}|$ in treatments B, C, and D were equivalent (500 V). Estimating the total energy delivered for treatments A, B, C, and D, $V_{hi}^2\tau_{on}$ is considered constant and, if the resistance is also considered constant and estimated from V–I measurements as R ≈ 150 Ω, similar applied energies were used for each treatments B, C, and D, with treatment A having a slightly lower applied energy due to the lower potential. The estimated energy dosage delivered during treatment A is estimated to be 0.12 J and 0.33 J for treatments B, C, and D. These estimates are corroborated by the measured potentials (Fig. 2). To estimate the worst-case Joule heating experienced by the cells during treatments B, C, and D, we assume that the energy delivered to the cells