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Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses

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ABSTRACT

Multiple studies have shown that bipolar (BP) electric pulses in the microsecond range are more effective at permeabilizing cells while maintaining similar cell survival rates as compared to monopolar (MP) pulse equivalents. In this paper, we investigated whether the same advantage existed for BP nanosecond-pulsed electric fields (nsPEF) as compared to MP nsPEF. To study permeabilization effectiveness, MP or BP pulses were delivered to single Chinese hamster ovary (CHO) cells and the response of three dyes, Calcium Green-1, propidium iodide (PI), and FM1-43, was measured by confocal microscopy. Results show that BP pulses were less effective at increasing intracellular calcium concentration or PI uptake and cause less membrane reorganization (FM1-43) than MP pulses. Twenty-four hour survival was measured in three cell lines (Jurkat, U937, CHO) and over ten times more BP pulses were required to induce death as compared to MP pulses of similar magnitude and duration. Flow cytometry analysis of CHO cells after exposure (at 15 min) revealed that to achieve positive FITC-Annexin V and PI expression, ten times more BP pulses were required than MP pulses. Overall, unlike longer pulse exposures, BP nsPEF exposures proved far less effective at both membrane permeabilization and cell killing than MP nsPEF.

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1. Introduction

Cell permeabilization is traditionally accomplished by application of micro- and millisecond monopolar (MP) electric pulses at electric field amplitudes of hundreds to thousands of volts per centimeter [1,2]. High-amplitude exposures cause irreversible electroporation (IRE), resulting in cell death, which has been used successfully *in vivo* to kill unwanted tissue [3,4]. Symmetric bipolar (BP) pulses, which are distinguished by a reversal of polarity halfway through pulse duration, have been investigated as a method to improve permeabilization of cell membrane by efficiently porating both sides of the cell. Tekle et al. showed that 400 μs BP pulses increased transfection efficiency while reducing cell death [5]. Related work by Kotnik et al. studied the impact of 1000μs (total duration) MP and BP pulses on the permeabilization of cells to bleomycin, survival, and uptake of Lucifer yellow [6]. Results showed that BP pulses both equivalent (500 + 500 μs) and double duration (1000 + 1000 μs) increased the permeabilization of cells to bleomycin and Lucifer yellow, but had the same effect on survival. They concluded that BP pulses offer the advantage of increased cell permeabilization without the downside of increased cellular mortality. Furthermore, the use of BP pulses for *in vivo* brain tissue ablation (IRE-based) was shown to be advantageous for reducing muscle contractions despite requiring higher-amplitude exposure to achieve a similar lethal effect [7]. Cleaner regions of ablation and more defined survival borders were also seen with BP IRE. Similar reduction in muscle contraction and maintained permeabilization efficiency was observed during electrochemotherapeutic treatment of skin cancer with BP pulses [8]. In a recent theoretical paper, Arena et al. predicted that pulses with polarity shifts in the nanosecond range would be advantageous in limiting joule heating and penetrating epithelial layers, resulting in more efficient electroporation of underlying tissues [9]. Taken together, these studies suggest an advantage to using BP pulses for electropermeabilization and IRE.

Nanosecond pulsed electric fields (nsPEF) have been shown to permeabilize the plasma membrane (PM), albeit with a larger population of smaller pores (i.e. nanopores), and have been speculated

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to produce intracellular permeabilization due to inherently higher frequency composition and faster rise times [10–14]. However, few papers have examined whether exposing cells to a BP nsPEF will offer the same advantages as observed with longer pulses. Vernier et al. used bursts of extremely short MP and BP nsPEF (15 ns, 50-pulses 28-kV/cm) to qualitatively show with FM1-43 that membrane, reorganization occurred symmetrically with BP pulses and asymmetrically with MP pulses [15]. However, little further assessment of the effectiveness of the exposure was presented. French et al. also showed that extremely short (total duration 1.6 ns at full width half maximum) BP (capacitively-coupled) pulses were less effective than MP (conductively-connected) pulses at permeabilizing cells, as measured by bleomycin uptake [16]. Given such limited data on BP nsPEF exposures and the potential advantages for *in vitro* and *in vivo* nsPEF applications, we measured the cellular impact of 600-ns BP nsPEF exposures as compared to MP exposures.

2. Materials and methods

2.1. Cell lines and propagation

Chinese Hamster Ovarian-K1 (CHO), U937, and Jurkat cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). CHO cells were propagated at 37 °C with 5% CO_2 in air, in F12K medium supplemented with 10% fetal bovine serum, 2-mM L-glutamine, and 100 IU/ml penicillin and 0.1 μg/ml streptomycin. Jurkat and U937 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2-mM L-glutamine, and 100-U/mL penicillin/streptomycin. The media and its components were purchased from Mediatech Cellgro.

2.2. Microscope exposure system

Monopolar pulses were delivered to the cells as described in previous publications [11]. A custom bipolar (BP) pulse generator was constructed to generate bipolar pulses using a full-bridge voltage-source inverter (VSI) consisting of four MOSFETs (IXYS, IXFB38N100Q2, 1 kV, Milpitas, CA). Either the top or the bottom switch of each leg was turned to allow generation of one polarity of the pulse. Timing was controlled by a function generator (Stanford, DG535, Sunnyvale, CA), which generated two control pulses to trigger the diagonal switches sequentially. Single MP and BP exposures were delivered to cells using a pair of tungsten electrodes (125-μm diameter, 135-μm separation) positioned 50-μm above the glass surface by a micromanipulator (Sutter MP285, Novato, CA). The BP and MP pulse shapes are shown in Fig. 1A along with their Fast Fourier Transform (FFT) spectrum (Fig. 1B) as computed by MATLAB® (Mathworks, Natlick, MA). Finite Difference Time Domain (FDTD) modeling, as in previous publications, predicted the resulting electric field amplitude for MP exposures to be 1.8–14 kV/cm at the cell. BP exposures ranged from 3 to 24 kV/cm. The amplitude of the electric field was predicted from the single phase peak voltage for both MP and BP exposures. Different electric fields were achieved by lowering the charging voltage feeding the pulser (1, 0.75. 0.5, 0.25, 0.125 kV, 0 kV). Due to difference between the MP and BP pulser circuitry, the resultant single phase pulse amplitude for a 1 kV charging voltage was roughly 480 V for the MP and 700 V for the BP pulses. This difference resulted in higher field exposures being generated for the same charging voltage for the BP exposures.

2.3. Fluorescence staining

For microscopy experiments, CHO cells were trypsinized and plated onto poly-L-lysine-coated 35-mm dishes with a glass coverslip bottom (MatTek, Ashland, MA). To stain cells, culture medium was removed, cells were twice washed with calcium free and magnesium free Dulbecco's phosphate buffered saline ((DPBS), Gibco, Grand Island, NY). A buffered solution containing 135-mM NaCl, 5-mM KCL, 10-mM HEPES, 10-mM Glucose, 2-mM $CaCl_2$, and 2-mM $MgCl_2$ with a pH of 7.4 and osmolality of 290–310 mOsm/kg was then placed on the cells (Sigma, St. Louis, MO). Calcium Green-1 AM ester (Molecular Probes, Eugene, OR) was added at a final concentration of 3-μM and incubated at room temperature for 30-min to allow for cellular uptake. Prior to imaging, cells were washed again with the buffer and allowed to rest for an additional 30-min. At the beginning of the experimentation, 3 μM propidium iodide (PI) was added to the cell dish. Due to spectral overlap, FM1-43 exposures were done independently by adding 9 μM FM1-43 (Molecular Probes, Eugene, OR) in deionized water to the cells in buffer. Cells were incubated with FM1-43 for 15-min prior to experimentation at room temperature.

2.4. Cuvette exposure system

To expose a large population of cells using an electroporation cuvette, CHO were removed from the culture dish with Trypsin–EDTA and suspended in fresh full F12K medium at a density of 1200-cells/μL (Gibco, Grand Island, NY). Jurkat and U937 cells were centrifuged and re-suspended in fresh media at a density of 1200-cells/μL. 90 μL of cells in a 1-mm aluminum electroporation cuvette were exposed to 10-kV/cm MP or BP pulses at 1-Hz repetition rate. Two Marx bank capacitor systems were used to generate either a 600-ns MP or 600-ns BP pulse. A high voltage power supply (0–10-kV/cm) was used to charge the Marx bank capacitors. Delivery to the cuvette was achieved by a spark gap switch that discharged over an air gap between two conductive plates completing the circuit. The rate of discharge and amplitude was set by adjusting the charging voltage and the distance between the plates. The pulse delivered to the cuvette was measured using a high voltage probe connected to a high speed oscilloscope (TDS3052B, Tektronix, Beaverton, OR). Removal of the charging voltage controlled the number of pulses delivered, which was counted manually. The resultant pulse shapes are shown in Fig. 1C along with their FFT spectrum (Fig. 1D).

2.5. MTT

Cell viability was measured at 24 h post exposure using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Gibco, Grand Island, NY). To perform the assay, 30 μL of exposed cells were transported to 96 well dishes containing 70 μL of fresh media, and incubated at 37 °C for 24 h. Then, 10 μL MTT reagent was added and incubated for 2. 100 μL detergent was added to each well, and the plate was left at room temperature for 2 h. The absorbance was then measured at 570 nm with a Synergy Plate Reader (Biotech).

2.6. Cell flow cytometry

Fluorescence and scattering measurements were made in CHO cells using an Acurri C-Flow flow cytometer (BD Biosciences, San Jose, CA). First, a staining solution containing fresh medium supplemented with 20-μL/ml of FITC-Annexin V (Molecule Probes, Eugene, OR) and 4-μL/mL PI (Molecule Probes, Eugene, OR) was made. Amber centrifuge tubes were filled with 50 μL of the staining solution to which 50 μL of exposed cells were added immediately after exposure. The contents were mixed gently using a 1-mL pipette and allowed to rest for 15-min at room temperature. Immediately before measurement, the cells were mixed to resuspend them evenly throughout the solution. The 50 μL cell solution