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Electropermeabilization by uni- or bipolar nanosecond electric pulses: The impact of extracellular conductivity

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ABSTRACT

Cellular effects caused by nanosecond electric pulses (nsEP) can be reduced by an electric field reversal, a phenomenon known as bipolar cancellation. The reason for this cancellation effect remains unknown. We hypothesized that assisted membrane discharge is the mechanism for bipolar cancellation. CHO-K1 cells bathed in high (16.1 mS/cm; HCS) or low (1.8 mS/cm; LCS) conductivity solutions were exposed to either one unipolar (300-ns) or two opposite polarity (300 + 300-ns; bipolar) nsEP (4–40 kV/cm) with increasing interpulse intervals (0.1–50 μs). Time-lapse YO-PRO-1 (YP) uptake revealed enhanced membrane permeabilization in LCS compared to HCS at all tested voltages. The time-dependence of bipolar cancellation was similar in both solutions, using either identical (22 kV/cm) or isoeffective nsEP treatments (12 and 32 kV/cm for LCS and HCS, respectively). However, cancellation was significantly stronger in LCS when the bipolar nsEP had no, or very short (<1 μs), interpulse intervals. Finally, bipolar cancellation was still present with interpulse intervals as long as 50 μs, beyond the time expected for membrane discharge. Our findings do not support assisted membrane discharge as the mechanism for bipolar cancellation. Instead they exemplify the sustained action of nsEP that can be reversed long after the initial stimulus.

1. Introduction

Permeabilization of the cell membrane by high voltage electric pulses (EP), also known as electroporation, has been well studied in recent decades [1–5]. Cells exposed to EP experience a buildup of charges across the plasma membrane. When the transmembrane potential (TMP) reaches a critical value, ~ 0.5–1 V, breakdown of the lipid bilayer occurs [1,5], allowing the transport of otherwise impermeant molecules across the membrane.

In spite of extensive research, the mechanism behind membrane permeabilization is an area of continued debate. One theory describes electropermeabilization as the formation of hydrophilic pores in the lipid bilayer [6,7]. The application of EP induces rearrangements of membrane components (including both lipids and water) to form an aqueous pore lined by lipid head groups. Support for the hydrophilic pore model has come primarily from molecular dynamics simulations [6–9] or indirect experimental evidence [10–12]. However, two recent studies directly visualized discrete electropore transport in planar droplet interface bilayers using optical single channel recordings [13,14]. Though the studies were not conducted in cells, their findings provide strong and convincing evidence for the formation of pores in the plasma membrane by EP. In a parallel hypothesis, leaky regions of the membrane are created by either destabilization of the lipid bilayer and/or peroxidation [15–17]. In fact, atomic force microscopy performed on live cells showed that the membrane elasticity was decreased by 40% after EP exposure [18]. In actuality, the mechanism of electropermeabilization likely involves a combination of multiple phenomena, in which the lipid bilayer becomes leaky and hydrophilic pores are formed. A recent study proposed a broader model of electropermeabilization, referring instead to the electropermeome, which includes additional cellular processes or structures, such as membrane proteins [19]. Nonetheless, for simplicity and convenience, we use the terminology “pore” here, with the understanding that permeabilization may in fact be more complex.

In the last decade, a number of studies have explored the biological effects of nanosecond duration EP (nsEP) [4,20–28]. As the duration of EP shortens (from micro- and milliseconds to nanoseconds), the buildup of membrane potential in cells shifts from Maxwell-Wagner polarization to other mechanisms, such as dielectric stacking [29]. Consequently, nsEP can target intracellular membranous structures,

Abbreviations: EF, electric field; HCS, high extracellular conductivity solution; LCS, low extracellular conductivity solution; nsEP, nanosecond electric pulses; σ_e, extracellular conductivity; TMP, transmembrane potential; YP, YO-PRO-1.

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including mitochondria [21,26], endoplasmic reticulum [30,31], and nucleus [32], as well as form nanometer-sized pores in the plasma membrane [24,33,34]. Downstream effects of nsEP-induced permeabilization include diffusion of ions and molecules through electropores [24,33,35–37], mobilization of intracellular Ca^{2+} [31,38–41], disassembly of the cytoskeleton [22], cell swelling and blebbing [42,43], and apoptotic or necrotic cell death [44–48], or autophagy [49].

We recently reported a phenomenon that is unique to nsEP, termed bipolar cancellation [50–52]. Unlike with micro- and millisecond bipolar pulses, reversing the polarity of the electric field (EF) can greatly reduce or eliminate the effects of the nsEP treatment. This cancellation of effects occurs either when the EF reversal is within a single bipolar pulse or by a pair of unipolar pulses of opposite polarity, despite delivering twice the energy and being twice as long in duration. Furthermore, cancellation of effects happens even when the second opposite polarity phase is only 35% of the first phase [52]. Hence, bipolar nsEP have proven to be far less efficient than unipolar pulses using diverse endpoints (including membrane permeabilization, Ca^{2+} mobilization, and cell death) and nsEP treatments. The fact that nsEP-induced effects can be undone after the delivery of the initial stimulus suggests a sustained and continuing action of the pulse after the nsEP treatment.

The mechanism(s) responsible for bipolar cancellation remains unknown. One hypothesis, which was supported by a theoretical analysis [53], involves the reverse electrophoretic transport of Ca^{2+} ions across the plasma membrane and out of the cell [51]. However, in a recent study, we demonstrated that bipolar cancellation occurred when Ca^{2+} was buffered both in the medium and cytosol, and thus did not necessarily depend on the reversal of electrophoretic flows of Ca^{2+} ions [52]. An alternative hypothesis proposes that an EF reversal facilitates the membrane discharge, thereby shortening the time that the membrane is above the critical voltage for permeabilization [51]. This mechanism has been termed assisted membrane discharge.

It is assumed that the passive discharge of the plasma membrane occurs with a time constant, τ_m, similar to that of membrane charging, and is around 0.1–1 μs for a typical mammalian cell [54]. Membrane charging time is influenced by many factors that are intrinsic to the cell, including cell radius, intracellular conductivity, membrane capacitance, and membrane conductance (see Section 2.2) [55]. In addition to these, the charging time constant is also affected by extracellular conductivity (σ_e), increasing as σ_e decreases. Likewise, the same dependence on extracellular conductivity may apply to the discharge of the membrane, whereby decreasing σ_e prolongs membrane discharge.

Several theoretical and experimental studies suggest that the membrane reaches the critical breakdown voltage of ~1 V within only a few nanoseconds, at which point it becomes clamped [28,37,56,57]. The time for this to occur is several orders of magnitude faster than the typical membrane charging time, making the influence of membrane conductivity less obvious. However, if we consider that a decrease in extracellular conductivity prolongs membrane discharge, the TMP would remain above the critical voltage for a longer duration, yielding more efficient permeabilization. Indeed, several groups demonstrated this effect, in which a decrease in σ_e resulted greater electropermeabilization [58–65]. On the other hand, nearly the same number of studies reported the opposite – no change or a decrease in the efficiency of the EP treatment as the extracellular conductivity decreased [55,66–70]. Only four of the above-mentioned studies evaluated the influence of σ_e on nsEP-induced permeabilization. The first study was from the Zimmermann group [61] and reported greater electropermeabilization by a single nsEP (durations from 10 to 100 ns, amplitudes up to 150 kV/cm) as σ_e decreased. The next two studies both came from the Mir group [55,63], and used the same duration nsEP and experimental approach, but presented opposite conclusions. In the earlier report [55], cells treated with 100 to 1000, 12-nsEP at 32 kV/cm (10 Hz) exhibited greater permeabilization in high conductivity media. On the contrary, in the subsequent study [63], they delivered 100-fold fewer 12-ns pulses at 4-fold higher EF amplitudes (1–10 pulses, 10 Hz, 142 kV/cm) and observed the opposite tendency. Finally, in a recent study [65], CHO-K1 cells were permeabilized to a greater extent in low σ_e following exposure to 300-, 600-, or 900-ns EP. Consequently, the influence that σ_e has on plasma membrane electropermeabilization remains poorly understood.

In this study, we tested the hypothesis that assisted membrane discharge is responsible for the bipolar cancellation phenomenon. We evaluated the cancellation effect of bipolar nsEP treatments with increasing intervals between the two opposite polarity pulses, and in different extracellular conductivity solutions. From our previous study, we know that as the interpulse interval increases, the cancellation effect decreases [51]. Decreasing the conductivity should prolong membrane discharge, thus increasing the time interval during which a polarity reversal can cancel the effects from the initial stimulus. Here we show that cancellation of effects can occur up to 50 μs after the first pulse, longer than the time expected for membrane discharge. Additionally, we found that the time-dependence of bipolar cancellation was similar between the two σ_e solutions. Hence, our results do not support the assisted membrane discharge hypothesis as the mechanism for bipolar cancellation. Rather, they exemplify the sustained and continuing action of nsEP that can still be reversed tens of microseconds after the initial stimulus.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO-K1) cells were maintained in F-12 K medium (Mediatech Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (certified OneShot FBS, Life Technologies, Grand Island, NY) and 100 IU/mL penicillin, and 0.1 μg/mL streptomycin (Mediatech Cellgro) at 37 °C, 5% CO_2. Approximately one day prior to experiments, cells were seeded on glass coverslips coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO) to improve cell adhesion. All experiments were performed at room temperature (22 ± 2 °C).

2.2. Exposure solutions

Cells were exposed to nsEP in either a high conductivity physiological solution (16.1 mS/cm; HCS) or a low conductivity solution (1.8 mS/cm; LCS). The conductivity of the solutions was measured with an ECTestr High conductivity meter (Oakton Instruments, Vernon Hills, IL). The high conductivity solution contained (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 Na-EGTA, 10 HEPES, 10 Glucose (pH 7.4 with NaOH). The recipe for the low conductivity solution was the same, except 135 mM NaCl was replaced with 280 mM adonitol. All chemicals and solutions were purchased from either Sigma-Aldrich or Life Technologies. The osmolality of both solutions was between 290 and 310 mOsm/kg, as measured with a freezing point Advanced™ Micro Osmometer (Model 3300, Advanced Instruments, Inc., Norwood, MA). Each solution was supplemented with 1 μM YO-PRO-1 (YP). The membrane charging time constant (τ_m) in each solution was calculated as in [55]:

$$\tau_m = r_c C_m \frac{\sigma_i + 2\sigma_e}{2\sigma_e\sigma_i + r(\sigma_i + 2\sigma_e)S_0} \quad (1)$$

where r is the cell radius (7 μm), C_m is the membrane capacitance (0.01 F/m^2), σ_i is the intracellular conductivity (5 mS/cm), σ_e is the extracellular conductivity (16.1 or 1.8 mS/cm), and S_0 is the initial conductance of the membrane (19 mS/cm^2).

2.3. NsEP exposure and electric field distribution

In this study, we used 300-ns duration pulses as a continuation from our previous work [51]. Nearly rectangular 300-ns EP were delivered to