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Effects of nanosecond pulse electric fields on cellular elasticity

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Abstract

We investigated the effects of a single 60 nanosecond pulsed electric field (nsPEF) of low (15kV/cm) and high (60 kV/cm) field strengths on cellular morphology and membrane elasticity in Jurkat cells using fluorescent microscopy and atomic force microscopy (AFM). We performed force displacement measurements on cells using AFM and calculated the Young's modulus for membrane elasticity. Differential effects were observed dependent upon pulsing conditions. We found that a single nsPEF of low field strength did not induce any apparent cytoskeletal breakdown and had minor morphological changes. Interestingly, force measurements and calculation of Young's modulus showed a significant decrease in membrane elasticity. A single nsPEF of high field strength induced stark morphological changes due to disruption of the actin cytoskeleton and a marked decrease in elasticity likely caused by irreversible membrane damage. We suggest that the cellular morphology is mainly dependent on stabilization by the actin cytoskeleton, while the elasticity changes are partially dependent on the cytoskeletal integrity.

Keywords

AFM; Young's Modulus; Nanosecond pulsed electric fields; Jurkats; cytoskeleton

1. Introduction

The application of electric fields across a biological membrane, causing lipid rearrangements called pores to form, is referred to as electroporation (EP). Depending on the electric field strength, pulse number and duration, the membrane effects can be temporary and will not induce cell death (Neumann and Rosenheck, 1972; Schoenbach et al., 2001). Conventional EP typically consists of pulses with duration greater than 100 μ s and sub-kV/cm voltages resulting in the formation of large heterogeneous pores in the plasma membrane (Gabriel and Teissie, 1999; Gowrishankar and Weaver, 2006; Tekle et al., 1990).

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Cortical actin destabilization was shown to not be related to electroporation of the cell membrane under long pulse durations (Chopin et al., 2013; Louise et al., 2014).

Nanosecond pulsed electric fields (nsPEFs) are characterized as high voltage (greater than 10 kV/cm), short duration (sub- μ s) electrical pulses capable of inducing cellular effects different from conventional EP. nsPEF-related effects include the creation of dense, homogenous nanopores in the cell plasma membrane and pore formation in intracellular membranes, such as organelles and the nuclear envelope (Schoenbach et al., 2001; Gowrishankar et al., 2006; Pakhomov et al., 2009). nsPEFs permeabilize the cell membrane without the uptake of propidium iodide (Pakhomov et al., 2007), unlike conventional EP, and is associated with an increase of intracellular calcium from both intracellular stores and extracellular sources (White et al., 2004; Semenov et al., 2013). The formation of nanopores and penetration into intracellular structures is attributed to pulse durations shorter than the charging time of the cell membrane (Schoenbach et al., 2001; Pakhomov et al., 2007). The permeabilization of external and internal membranes is thought to be the main mechanism of cell death. Previously, we observed that nsPEFs are able to induce chromosome and telomere damage and actin cytoskeleton disruption which was found to also contribute to cell death (Stacey et al., 2003; Stacey et al., 2011).

Many investigations have studied the nature of pore formation in cells subjected to nsPEFs while the morphological changes and overall membrane elasticity remain relatively unknown. In this study, we utilized atomic force microscopy (AFM) and fluorescent microscopy to determine the changes in cellular morphology and elasticity.

2. Methods

2.1 Cell Culture

Jurkat cells are an immortalized human T-lymphocyte cell line which grows in suspension. Jurkat clone E6-1 (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Atlanta Biologicals, Norcross, GA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine (Gibco/Invitrogen, Waltham, MA, USA), 50 IU/ml penicillin (Gibco/Invitrogen), and 50 mg/ml streptomycin (Gibco/Invitrogen) at 37°C with 5% CO₂ in air. Cells were maintained in T75 flasks (Thermo Fisher Scientific, Waltham, MA, USA) and fresh medium was added every 2–3 days. Subculturing was performed when cells reached a high density ($\sim 3 \times 10^6$ cells/ml) and the suspension was centrifuged at $200 \times g$ for 5 minutes and cells resuspended at 1×10^5 .

2.2 Cell Electroporation and Preparation

For electroporation experiments, cuvettes with 1 mm gaps (BioSmith, San Clemente, CA, USA) were used which have a working volume of 150 μ L. The cell suspension was counted and harvested by centrifugation at $200 \times g$ for 5 minutes, and then resuspended in fresh medium at a concentration of 1×10^6 cells per 150 μ L.

The cells were placed in the cuvettes and exposed to a single pulse of 60 nanoseconds with pulse strengths of 0, 15, or 60 kV/cm. The pulses were generated using a Blumlein pulse generator as previously described (Schoenbach et al., 2001). After exposure, cells were

immediately fixed with 4% paraformaldehyde then transferred to poly-L-lysine coated coverslips (Sigma-Aldrich, St. Louis, MO, USA). After fixation, cells were washed three times in PBS for 5 minutes each and then imaged using atomic force microscopy, or the cytoskeleton stained for fluorescent microscopy..

2.3 Cytoskeletal Staining

For the cytoskeletal staining, following fixation and washing, the cells were incubated in Oregon Green 488 phalloidin (Gibco/Invitrogen) for 20 minutes to stain F-actin and then washed three times in PBS + 1% Tween-20 (PBS-T) for 5 minutes each. The nuclei were counterstained using 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) in PBS-T for 5 minutes followed by three PBS-T washes for 5 minutes each. After a brief rinse in PBS, the coverslips were mounted on slides using VECTASHIELD antifade mounting medium (Vector Labs, Burlingame, CA, USA). Electronic fluorescent images were captured using an Olympus DP70 CCD camera through an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA, USA).

2.4 Atomic Force Microscopy

The height, phase, and NSOM images of the Jurkat cells were obtained by using a Multiview-4000 multi-probe atomic force microscope (Nanonics Imaging, Jerusalem, ISR) as previously described (Stacey et al., 2013). In brief, the coverslips with fixed cells were loaded onto the AFM stage and images with a resolution of 256×256 pixels were obtained using NWS (Nanonics) and WSxM 5.0 (Nanotech Electronica, Madrid, ESP) software with a 20 nm parabolic quartz tip in tapping mode. The cantilever has a spring constant of 2600 µN/µm and resonance frequency of 33.97 kHz. Calibration was performed using a silicon grid and a profilometer. Force measurements were processed and verified using silicon and polydimethylsiloxane (PDMS).

2.5 Young's Modulus

To calculate the Young's modulus, a modified Hertz model known as the Derjaguin-Muller-Toporov (DMT) model was used (Derjaguin et al., 1975). The DMT model gives the stress distribution with an indentation depth δ in the area of contact using the equation below:

$$F_{tip} = \frac{4}{3} E^* \delta^{\frac{3}{2}} \sqrt{R} + F_{adh} \quad (1)$$

The applied load using a parabolic tip is F_{tip} where the apical radius is R , the indentation depth is δ , E^* is the relative Young's modulus, and F_{adh} is the adhesion force between the tip and the sample due to intermolecular forces. The relative Young's modulus is given by:

$$E^* = \frac{1}{\left(\frac{1-\nu_{tip}^2}{E_{tip}} + \frac{1-\nu_{sample}^2}{E_{sample}} \right)} \quad (2)$$

where the Young's modulus of the tip and sample are E_{tip} and E_{sample} , respectively, and the Poisson ratio of the tip and sample are ν_{tip} and ν_{sample} , respectively.

A least square curve fitting algorithm was used to extrapolate two parameters from Eq.1, the adhesion force and the relative Young's modulus. The Young's modulus was calculated and graphed using MATLAB (MathWorks, Natick, MA, USA) by solving for E_{sample} in Eq.2 using the relative Young's modulus from Eq.1. The calculated elasticity was calibrated using PDMS as a standard. Statistical analysis was performed using Student's *t*-test and $p < 0.05$ indicated a significant result.

3. Results

3.1 Actin and Morphology

The application of nsPEFs to cells is known to cause the formation of nanopores. Computational modeling and experimental studies have studied the size, density, and lifespan of these pores (Gowrishankar and Weaver, 2006; Pakhomov et al., 2009; Pakhomov et al., 2007; Vasilkoski et al., 2006). The effect of high energy, low duration electric fields on the membrane integrity and cytoskeleton is not well known. We investigated the cellular cytoskeleton with fluorescent microscopy. Using DAPI and Phalloidin, we were able to visualize the nucleus and actin cytoskeleton (Figure 1). Jurkat cells are suspension cells which have a round cell morphology. Untreated cells (0 kV/cm) display a nucleus with a relatively round, crisp cytoskeletal outline (Figure 1A–C). Following the application of a 15 kV/cm nsPEF, the actin cytoskeleton appears to be intact and appears similar to the untreated cells (Figure 1D–F). A 60 kV/cm nsPEF caused the actin cytoskeleton at the periphery of the cell to become more diffuse and some cells also lost their round shape (Figure 1G–I). The diffuse localization of actin and formation of foci suggests that the 60 kV/cm nsPEF induced major destabilization of the actin cytoskeleton.

3.2 AFM and Morphology

Using AFM and NSOM, the morphological changes associated with the application of different nsPEFs were visualized (Figure 2). The AFM data collected was used to produce the height and phase profiles. The height profile (Figure 2A–C) is representative of changes in topography from the physical interaction with the surface, while the phase profile is dependent on the height, hardness, and chemical makeup of the surface (Figure 2D–F). NSOM was used to visualize the morphology optically (Figure 2G–I). Using the AFM data collected, three-dimensional representations were also generated (Figure 2J–L). We found that a single 60 ns, 15 kV/cm pulse induced a small change in the morphology by changing the cell from a rounded (Figure 2J) to an oval shape (Figure 1K). The 60 kV/cm nsPEF had deleterious effects showing marked morphological changes (Figure 1L).

3.3 Young's Modulus

Using force spectroscopy mode atomic force microscopy, force-indentation measurements were taken and used to calculate the Young's modulus. The Young's modulus was calibrated with known values for PDMS. Our force-indentation measurements and calculated Young's modulus for PDMS was 550.33 (± 15.70) kPa (Figure 3), which is in agreement with the previously published value of 549 kPa (Armani et al., 1999). Measurement of the elasticity of the cells (Figure 4) found a 53% decrease in the Young's modulus when Jurkat cells were exposed to 15 kV/cm (Figure 4E and Table 1). No apparent

observed change to the actin cytoskeleton network under fluorescence microscopy suggests that the reduction in Young's modulus is due to factors other than the cytoskeleton, possibly the porosity of the cell membrane. However, the application of a 60 kV/cm nsPEF resulted in an 85% decrease of the Young's modulus at the maximum indentation depth when compared to 0 kV/cm (Figure 4E and Table 1). Taken together the changes observed to the actin cytoskeleton in Figure 1H, and given that the cytoskeleton network confers shape, rigidity, and elasticity to the cells, then the decreased Young's modulus with increasing electric field strengths suggests that nsPEFs disrupt the cytoskeleton resulting in decreased stiffness of the cell membrane.

4. Discussion

This is the first study measuring nanosecond pulsed electric field effects on membrane elasticity. We investigated the changes in the cellular morphology and elasticity of Jurkat cells due to a single 60 nanosecond pulsed electric field (nsPEF) of low (15kV/cm) and high (60 kV/cm) field strengths using fluorescent microscopy and atomic force microscopy (AFM). Fluorescent staining with phalloidin was used to visualize the filamentous actin and DAPI for nucleic acids. We performed force measurements on cells using AFM and calculated the Young's modulus by fitting our force-indentation measurements into a modified Hertz model known as the Derjaguin-Muller-Toporov (DMT) model. The Young's modulus is used to characterize the elastic modulus, or stiffness, of the cell membrane which is the ability to resist deformation. We also imaged the cellular morphology using near-field scanning optical microscopy complemented by AFM height and phase imaging.

The Jurkat cell line was chosen due to their growth in suspension with a round morphology, allowing for easier observation of morphological changes. Following a low field strength nsPEF application to Jurkats, the cells became more oval in shape (Figure 2K). Though phalloidin staining did not show any distinct changes in the actin cytoskeleton (Figure 1E), the elasticity was significantly reduced by 53% (Figure 3E). This suggests that the low field strength nsPEF has a considerable effect on the cell membrane but not the actin cytoskeleton, in agreement with low field strengths experienced under long pulsing conditions (Chopin et al., 2013; Louise et al., 2014). The elasticity decrease seems to be independent of actin effects and likely due to lipid rearrangements. The high field strength nsPEF caused substantial changes in cell morphology (Figure 2L), disruption of the actin cytoskeleton (Figure 1H), and an 85% reduction in elasticity (Figure 3E). The depolymerization and disruption of actin filaments, possible destruction of the cell cortex (Berghofer et al., 2009), and lipid rearrangements when using high field strengths are likely tied to the extensive morphological and elasticity changes.

5. Conclusion

In conclusion, our results show that nanosecond pulsed electric fields induce substantial changes in the cell elasticity. Specifically, we observed significant decreases in the membrane elasticity using both low and high field strengths, while actin destabilization was apparent with high field strength. The observed effects are likely caused by long-lasting permeabilization of the membrane as the measurements were performed on cells that were

fixed less than one minute of the application of the nsPEF. These long-lasting and even irreversible effects are partially dependent on the cell cytoskeleton. The plasma membrane interacts and is stabilized by the cell cytoskeleton (Berghofer et al., 2009). The destabilization of the actin cytoskeleton would result in further loss of membrane integrity and aberrant cellular morphology which was observed using nsPEFs with a high field strength (60kC/cm, Figure 1H and 2L). This is supported by our previous findings where disruption of actin before the application of nsPEFs resulted in a significant decrease in the survival of cells (Stacey et al., 2011). Using AFM, we were able to visualize and determine the level of membrane destabilization using force-indentation measurements. In the future, we intend to investigate the degree of stabilization that the actin cytoskeleton contributes to the plasma membrane when applying nsPEFs. The diverse effects of electric fields in biological systems is important, and nsPEFs provide a tool for extending the scope of these investigations as they predominantly affect internal cell structures compared to long pulse conditions.

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HIGHLIGHTS

- NsPEFs induce differential cellular effects in the Young's modulus measured by AFM
- Low and high nsPEF field strengths both significantly decrease membrane elasticity
- High field strength nsPEFs effect both membrane and actin cytoskeleton

Figure 1. Actin analysis of cells exposed to nanosecond pulsed electric fields (nsPEF) using fluorescent microscopy. DAPI (blue) staining of nucleic acids, Phalloidin (green) staining of F-actin, and merged images of Jurkat cells exposed to a single 60 ns pulse of (A–C) 0 kV/cm, (D–F) 15 kV/cm and (F–I) 60 kV/cm.

Figure 2.

Atomic force microscopy (AFM) and near-field scanning optical microscopy (NSOM) of Jurkat cells exposed to nanosecond pulsed electric fields (nsPEFs). (A–C) AFM height images of cells exposed to 0, 15, and 60 kV/cm nsPEFs, respectively. (D–F) NSOM images of Jurkat cells exposed to 0, 15, and 60 kV/cm nsPEFs, respectively. (G–I) AFM phase images of Jurkat cells exposed to 0, 15, and 60 kV/cm nsPEFs, respectively. (J–L) Three-dimensional representations based on AFM height measurements of Jurkat cells exposed to 0, 15, and 60 kV/cm nsPEFs, respectively.

Figure 3.
Force-indentation measurements of PDMS using force spectroscopy mode atomic force microscopy.

Figure 4.

Young's modulus of Jurkat cells exposed to nanosecond pulsed electric fields (nsPEFs) using force spectroscopy mode atomic force microscopy. Membrane force measurements of cells exposed to (A) 0, (B) 15, and (C) 60 kV/cm nsPEFs using FS-AFM. The black circles mark the point on the cell where force measurements were taken. Comparison of (D) force measurements and (E) Young's modulus in cells pulsed for 60 ns at 0, 15, and 60 kV/cm ($n = 3$ for all conditions).

Table 1

Calculated Young's Modulus

Pulse Strength	0 kV/cm	15 kV/cm	60 kV/cm
Mean ($\pm$ SD)	7.27 ($\pm$ 1.07)	3.40 ($\pm$ 1.19)	1.09 ($\pm$ 0.395)
% Reduction from 0 kV/cm	---	53.3% **	85.0% ***

Calculated Young's Modulus given in kPa.

** Statistically significant ($p < 0.01$)

*** Statistically significant ($p < 0.001$)