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14. ABSTRACT
Cells exposed to nanosecond-pulsed electric fields (nsPEF) exhibit a wide variety of nonspecific effects, including blebbing, swelling, intracellular calcium bursts, apoptotic and necrotic cell death, formation of nanopores, and depletion of phosphatidylinositol 4,5-biphosphate (PIP2) to induce activation of the inositol trisphosphate/diacylglycerol pathway. While several studies have taken place in which multiple pulses were delivered to cells, the effect of pulse repetition rate (PRR) is not well understood. To better understand the effects of PRR, a laser scanning confocal microscope was used to observe CHO-K1 cells exposed to ten 600ns, 200V pulses at varying repetition rates (5Hz up to 500KHz) in the presence of either FM 1-43, YO-PRO-1, or Propidium Iodide (PI) fluorescent dyes, probes frequently used to indicate nanoporation or permeabilization of the plasma membrane. Dye uptake was monitored for 30 seconds after pulse application at a rate of 1 image/second. In addition, a single long pulse of equivalent energy (200V, 6 □s duration) was applied to test the hypothesis that very fast PRR will approximate the biological effects of a single long pulse of equal energy. Upon examination of the data, we found strong variation in the relationship between PRR and uptake in each of the three dyes. In particular, PI uptake showed little frequency dependence, FM 1-43 showed a strong inverse relationship between frequency and internal cell fluorescence, and YO-PRO-1 exhibited a "threshold" point of around 50 KHz, after which the inverse trend observed in FM 1-43 was seen to reverse itself. Further, a very high PRR of 500 KHz only approximated the biological effects of a single 6 □s pulse in cells stained with YO-PRO-1, suggesting that uptake of different dyes may proceed by different physical mechanisms.

15. SUBJECT TERMS
nsPEF, nanoporation, pulsed electric fields, pulse repetition rate, fluorescence microscopy, confocal microscopy

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High frequency application of nanosecond pulsed electric fields alters cellular membrane disruption and fluorescent dye uptake

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ABSTRACT

Cells exposed to nanosecond-pulsed electric fields (nsPEF) exhibit a wide variety of nonspecific effects, including blebbing, swelling, intracellular calcium bursts, apoptotic and necrotic cell death, formation of nanopores, and depletion of phosphatidylinositol 4,5-biphosphate (PIP2) to induce activation of the inositol trisphosphate/diacylglycerol pathway. While several studies have taken place in which multiple pulses were delivered to cells, the effect of pulse repetition rate (PRR) is not well understood. To better understand the effects of PRR, a laser scanning confocal microscope was used to observe CHO-K1 cells exposed to ten 600ns, 200V pulses at varying repetition rates (5Hz up to 500KHz) in the presence of either FM 1-43, YO-PRO-1, or Propidium Iodide (PI) fluorescent dyes, probes frequently used to indicate nanoporation or permeabilization of the plasma membrane. Dye uptake was monitored for 30 seconds after pulse application at a rate of 1 image/second. In addition, a single long pulse of equivalent energy (200V, 6 μs duration) was applied to test the hypothesis that very fast PRR will approximate the biological effects of a single long pulse of equal energy. Upon examination of the data, we found strong variation in the relationship between PRR and uptake in each of the three dyes. In particular, PI uptake showed little frequency dependence, FM 1-43 showed a strong inverse relationship between frequency and internal cell fluorescence, and YO-PRO-1 exhibited a "threshold" point of around 50 KHz, after which the inverse trend observed in FM 1-43 was seen to reverse itself. Further, a very high PRR of 500 KHz only approximated the biological effects of a single 6 μs pulse in cells stained with YO-PRO-1, suggesting that uptake of different dyes may proceed by different physical mechanisms.

Keywords: nsPEF, nanoporation, pulsed electric fields, pulse repetition rate, fluorescence microscopy, confocal microscopy

1. INTRODUCTION

Nanosecond pulsed electric fields (nsPEF), defined as large magnitude electric fields of less than one microsecond duration, have become a popular area of study in recent years. Bioeffects associated with nsPEFs include nanoporation (the formation of nanometer-scale, transient pores in cellular membranes), PIP2 depletion, calcium spikes, inositol triphosphate production, cellular swelling and blebbing, and apoptotic and necrotic cell death[1-3]. Throughout the literature, studies exist which examine the effects of varying physical exposure conditions such as pulse duration, field intensity, and pulse number. In many of these cases, multiple cell lines have been studied using varied techniques including survivability assays, fluorescence microscopy, and genomic analysis[4]. A more recent avenue of investigation has been an attempt to discover the effects of multiple nsPEF applications, and interesting phenomena has been uncovered relating to pulse polarity[5, 6]. However, one particularly underrepresented aspect of these applications is the frequency that electric pulses are applied, known as pulse repetition rate (PRR). To our knowledge, only one study has attempted to quantify these effects, and experiments were limited to frequencies of less than 100 Hz[7]. In this study, we examine Chinese Hamster Ovarian (CHO) cells exposed to varying frequencies of 10, 600 nanosecond pulses across several orders of magnitude (5 Hz up to 500 KHz). In addition, we examined a single pulse of equivalent duration and energy (6 μs) in order to determine whether very high repetition rates in the hundreds of KHz can approximate an individual longer pulse. To quantify the bioeffects associated with these

exposures, laser scanning confocal microscopy was performed using a variety of common fluorophores (FM 1-43, YO-PRO-1, and Propidium Iodide) possessing different hydrated molecular sizes, as well as common pharmaceuticals (Ruthenium Red, Gadolinium) used in the study of nanoporation effects. Fluorescence intensities were quantified using ImageJ software and plotted as a function of time to determine trends across both fluorophores and the frequency spectrum.

2. METHODOLOGY

2.1 Cell Culture

Chinese Hamster Ovarian cells (CHO-K1 line), transfected with a human muscarinic type 1 receptor (hM_1), were chosen to provide continuity with prior experiments, and were prepared using standard procedures. The culture medium contained Ham's F-12K media (Life Technologies), 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/streptomycin, Life Technologies). In addition, 0.48% G418 antibiotic mixture (Life Technologies) was included for transfection stability. Prior to microscopy (~18 hours) cells were plated on poly-D-lysine coated, 35mm glass-bottomed dishes (MatTek No. 0, Ashland, MA), and left in a standard incubator overnight to allow for unperturbed cells for imaging.

2.2 Pulsing System

Delivery of the nanosecond pulses was accomplished via a pair of tungsten filaments (0.005" diameter) submerged in the imaging buffer and insulated at all points except for a small area at the tip. For consistent field delivery, a micromanipulator was used to position the probes exactly 50 microns above the glass imaging window. The probe filaments were also coupled to an oscilloscope to verify pulse shape and amplitude. To create the pulses, a high power Velonix pulse generator (Velonix, Model 360) with external pulse repetition rate controls was used. The pulse was triggered using an HP pulse generator, described below.

The experimental timing was set using a Digital Delay and Pulse Generator (Stanford Research Systems, Model DG535), including activation of the microscope and high voltage pulser. Upon activation, the Stanford system triggered the Zeiss LSM 710 microscope to initiate image acquisition using both bright field and laser scanning modes, with appropriate excitation and emission filters with regard to the fluorophore being used. After acquiring a baseline for 5 seconds, the Stanford was set to trigger a pulse generator (Model HP 8112A) which ensured an accurate and appropriate PRR for each exposure. This pulse generator was coupled to the Velonex high power pulse generator via a BNC coaxial cable, which could then deliver the appropriate pulse amplitude, repetition rate, and shape to the cells via the tungsten electrode pair. The final pulse energy output characteristics from the Velonex was viewed in real time on a Tektronix TDS3052 oscilloscope, and a voltage dividing probe was used to appropriately couple the high-voltage signal without damaging the oscilloscope.

2.3 Fluorescence Imaging

Approximately 18 hours prior to exposure, cells were plated on poly-D-lysine coated optical imaging dishes and left to incubate overnight in their standard growth medium. The next morning, this media was aspirated, and cells were washed with a standard imaging buffer. The dishes were refilled with buffer (3 mL) containing the appropriate concentration of one fluorophore (2mM YO-PRO-1, 3mM Propidium Iodide, or 8mM FM 1-43). In further experiments examining the effects of pharmaceutical agents, YO-PRO-1 was used as the fluorophore due to the strength of signal it provided. In these cases, the buffer solution was supplemented using either 50μM Ruthenium Red or 300μM Gd^{3+} to block certain families of calcium channels and/or nanopores. All experiments were performed within 45 minutes of removing cells from their incubator environment.

2.4 Exposure Conditions and Data Analysis

Several pulse repetition rates, spanning six orders of magnitude, were used for this study. In particular, these rates were 5 Hz, 500 Hz, 50 KHz, 250 KHz, and 500 KHz. While close to a fundamental limit of overlapping 600ns pulses, 500 KHz was also chosen as the maximum PRR which could maintain the relatively square waveform emitted from the Velonex. Ten 600ns pulses were delivered in this manner, as well as a subset which delivered a single 6 μs pulse to treat as a "continuous" application of ten 600ns pulses. A sham exposure (no pulse) was also measured. For every exposure

condition, exposures were randomized and repeated (one of each exposure condition per plate) until between 20 and 40 cells were taken of every condition. This process was repeated for the YO-PRO-1 experiments involving Gadolinium and Ruthenium Red, although the number of conditions per plate was cut in half to prevent cellular apoptosis induction initiated by exposure to these agents over longer time. All images were acquired at a rate of one per second (5 before pulsing, 30 after) and imported into ImageJ for sectioning and analysis. Bright field exposures were used to trace cell borders by hand, and Mean Fluorescence in the cell interior was monitored over time for every cell.

3. RESULTS

Representative waveforms from the oscilloscope are seen in Figure 1. While a slight roll-off is observed during the 6 microsecond pulse which is not entirely square, the amplitude was set so that the average amplitude throughout the pulse duration was 200 volts.

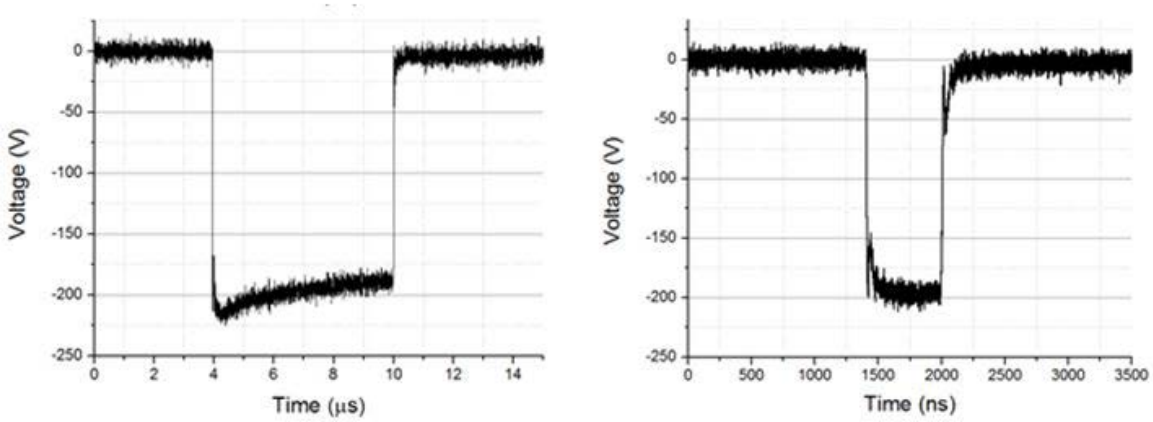

Figure 1. Sample Waveforms Delivered to Cells via the Tungsten Electrodes. (Left) Representative six microsecond pulse delivered to compare with very high PRRs. Note the slight roll-off in amplitude over time. (Right) Representative 600 ns pulse. Pulses of this approximate shape were delivered in groups of ten at repetition rates ranging from 5 Hz to 500 KHz. All pulse amplitudes were set to 200 volts.

Examining the results from the Propidium Iodide exposures (Figure 2) found very little frequency dependence observed across PRRs. Interestingly, very high exposure frequencies do not approach values similar to a "continuous" exposure of equivalent energy. Error bars in the plots represent standard error of the mean.

Examination of the FM 1-43 data shows a much cleaner trend (Figure 3). In practically every case, dye uptake is decreased as a function of PRR. However, in similar fashion to the propidium iodide exposures, a single long pulse creates a very different fluorescence response than ten high frequency pulses of the same energy. We should also note, in each of these conditions, sham exposures correctly produce no increase in fluorescence, and typically show a slight decrease due to photobleaching as expected from this dye.

Figure 4 shows the fluorescence response of YO-PRO-1 after pulsing. While the inverse trend observed in FM 1-43 is apparent up to 50 KHz, we actually observe a trend reversal past this point, with fluorescence intensities results actually showing increasing PRRs above 50 KHz. In this case only, our original hypothesis was correct; the fluorescence change observed at high frequencies appears to approach the result of a "continuous" application asymptotically. Because of the

strong signal and smaller error bars associated with YO-PRO-1, we used this dye to further investigate different exposure conditions.

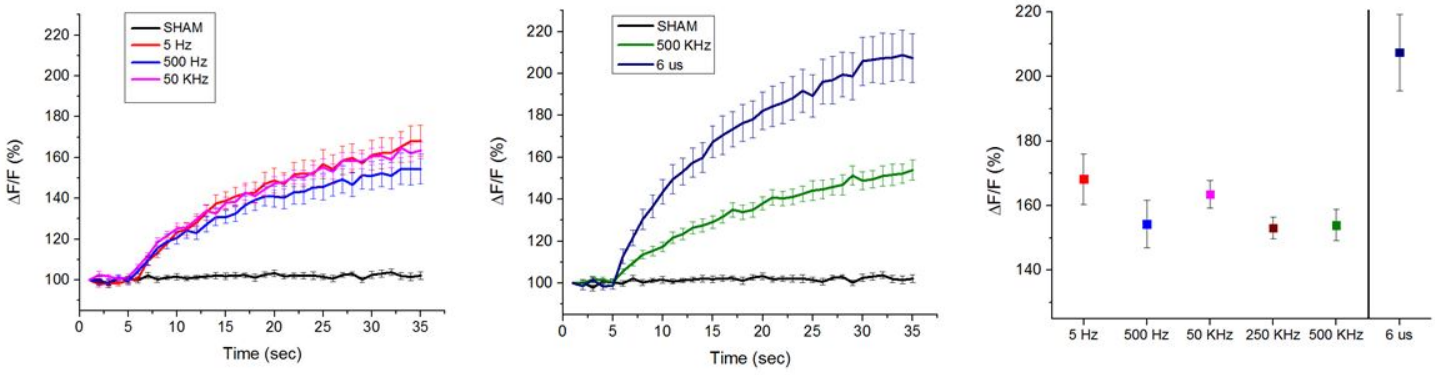

Figure 2. Fluorescence Increase over Time for Propidium Iodide Cell Exposures (Left) Fluorescence intensity measurements of propidium iodide over time of three representative pulsing frequencies (5, 500, and 50,000 Hz). (Center) Comparison of very high frequency (500 KHz) application of nsPEF with a single pulse of equivalent duration. (Right) Percent change in intensity measurement 30 seconds after initiation of pulses.

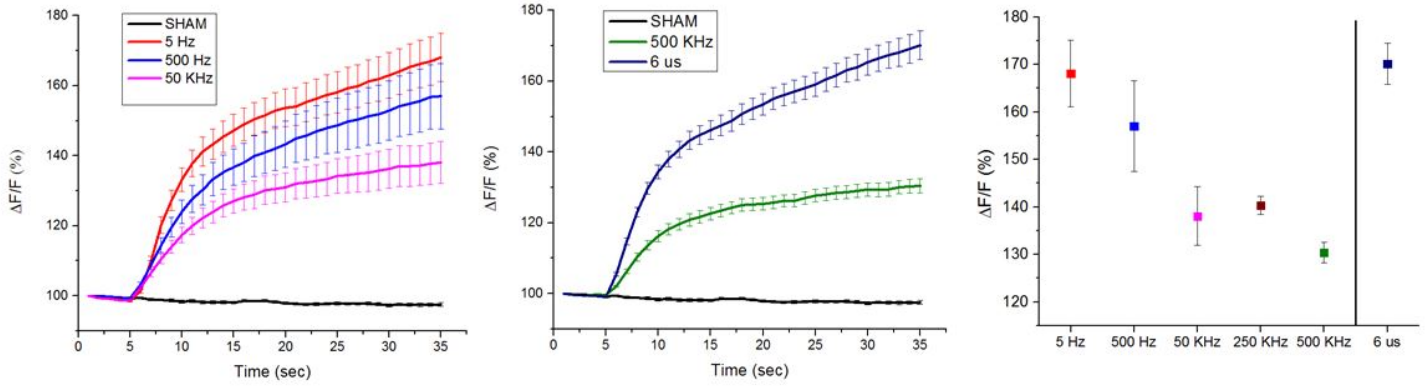

Figure 3. Fluorescence Increase over Time in Cells Stained using FM 1-43 Dye. (Left) Faster pulsing in general leads to a weaker dye uptake response throughout the exposure. (Center) A single six microsecond pulse is substantially more effective than ten 600ns pulses of equivalent energy. (Right) An inverse trend between PRR and fluorescence increase after 30 seconds is observed. A single 6 microsecond pulse is approximately as effective as ten pulses at 5 Hz.

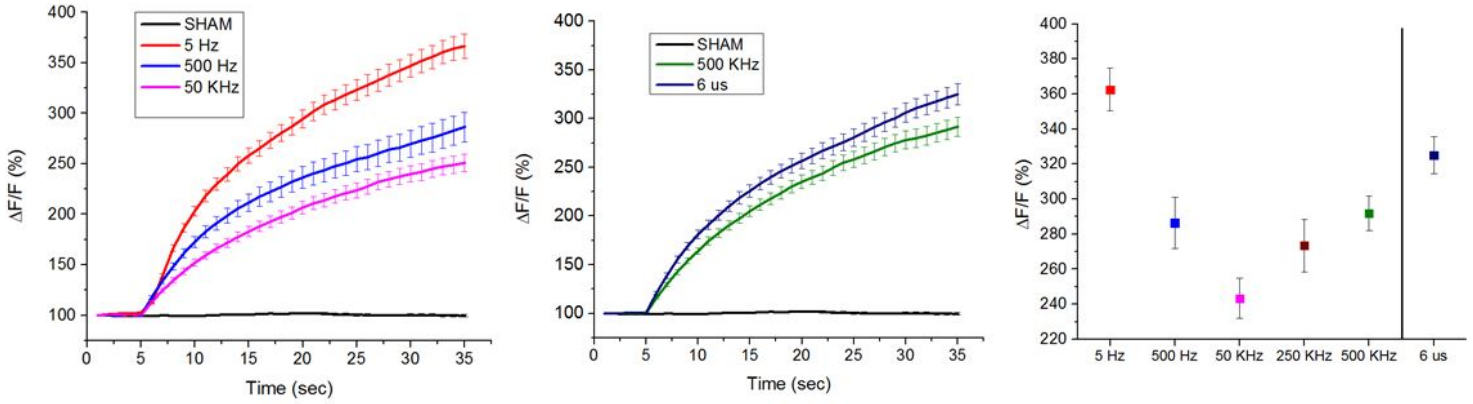

Figure 4. Fluorescence Increase over Time in Cells Stained using YO-PRO-1. (Left) Up to 50 KHz, the same inverse relationship between fluorescence increase and PRR is observed. (Center) 500 KHz PRR exposure is nearly as effective as a single long pulse using YO-PRO-1 staining. (Right) An interesting "thresholding" effect is observed after 50 KHz, in which the previously observed inverse law appears to reverse itself.

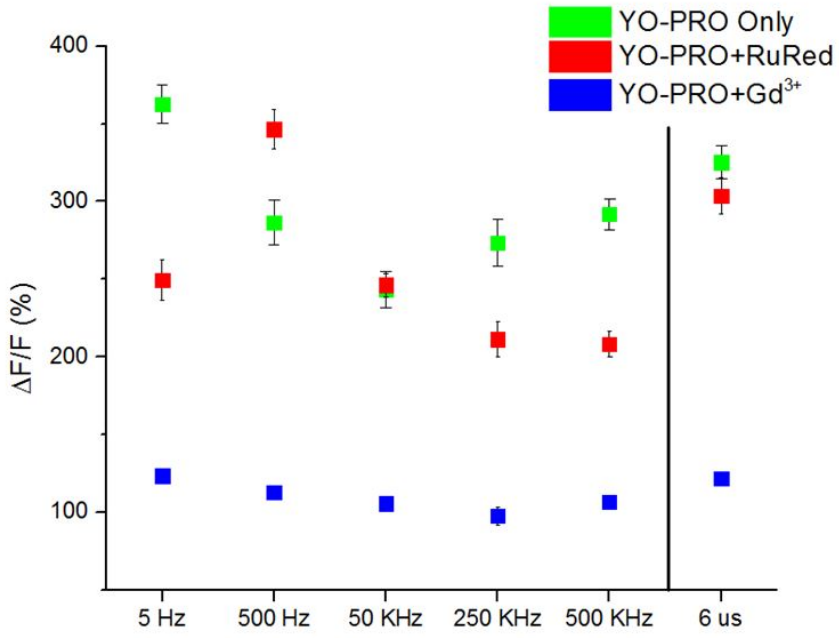

Figure 5. Cells Membrane Channels Probed using Ruthenium Red and Gadolinium. YO-PRO-1stainedcells were subjected to a range of PRRs in the presence of Ruthenium Red and Gadolinium, commonly used to study membrane channel activity. Gadolinium substantially reduces the fluorescence response (to near extinction) although the same trend reversal remains. Ruthenium Red, by contrast, substantially alters the frequency response.

In Figure 5, we plot the final fluorescence intensity across pulse repetition rates of cells stained with YO-PRO-1 in the presence of Ruthenium Red (red), Gadolinium (blue) or standard imaging buffer (green). Following closely with the literature, we observed a near total reduction in fluorescence in the presence of Gadolinium ions. However, the "thresholding" trend observed previously is still observed. Ruthenium Red (a semi-selective blocker of TRPV channels)

appears to alter the observed trend entirely providing a complex result that requires further study for effects interpretation.

The widely varying relationships between PRR and fluorescence observed in this study, especially across fluorophores, are indicative of chemical specificity in the uptake process. It is possible that protein channels (which exhibit chemical specificity themselves) are related to the uptake of dyes in an important and substantive way. While we used two known channel blockers in association with YO-PRO-1, there is a significant amount of work to be done to elucidate the roles that protein channels play across differing nsEP dosing rates. Recent research has also shown that mechanical stress may induce some or all of the biophysical effects associated with nanoporation[8-10]. The improvement of mechanical spectroscopies[11, 12] in recent years should prove useful for further investigation of these mechanisms.

4. CONCLUSIONS

This investigation provides some of the first knowledge examining the biological effects of varying high frequency nsPEF exposure in mammalian cells. Due to the pilot nature of the study design, a wide variety of PRRs, fluorophores, and exposure conditions were used to establish baselines for future work. Trends across pulse repetition rates were established which appear to change with the identity of the fluorophore in question, potentially indicating separate biological mechanisms for the uptake of individual fluorophores, and possibly implicating protein channels in a more substantive way than suggested by the current literature. In general, further work is needed to establish a link between the effects observed and presented in this proceeding and the biophysical mechanisms underlying these observations.

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