Published in final edited form as:

J Cardiovasc Electrophysiol. 2019 March ; 30(3): 392–401. doi:10.1111/jce.13834.

EXCITATION OF MURINE CARDIAC MYOCYTES BY NANOSECOND PULSED ELECTRIC FIELD

Jan E. Azarov, PhD^{1,2,3}, Iurii Semenov, PhD¹, Maura Casciola, PhD¹, and Andrei G. Pakhomov, PhD^{1,*}

¹Frank Reidy Research Center for Bioelectronics, Old Dominion University, 4211, Monarch Way, Norfolk, Virginia, USA, 23508

²Laboratory of Cardiac Physiology, Institute of Physiology, Komi Science Center, Ural Branch, Russian Academy of Sciences, 50, Pervomayskaya st., Syktyvkar, Russia, 167982

³Department of Physiology, Medical Institute of Pitirim Sorokin Syktyvkar State University, 11, Babushkin st., Syktyvkar, Russia, 167000

Abstract

Introduction.—Opening of voltage-gated sodium channels takes tens to hundreds of microseconds, and mechanisms of their opening by nanosecond pulsed electric field (nsPEF) stimuli remain elusive. This study was aimed at uncovering the mechanisms of how nsPEF elicits action potentials (APs) in cardiomyocytes.

Methods and Results.—Fluorescent imaging of optical APs (FluoVolt) and Ca^{2+} -transients (Fluo-4) was performed in enzymatically isolated murine ventricular cardiomyocytes stimulated by 200-ns trapezoidal pulses. nsPEF stimulation evoked tetrodotoxin-sensitive APs accompanied or preceded by slow sustained depolarization (SSD) and, in most cells, by transient afterdepolarization waves. SSD threshold was lower than AP threshold (1.26 ± 0.03 vs 1.34 ± 0.03 kV/cm, respectively, $p < 0.001$). Inhibition of L-type calcium and sodium-calcium exchanger currents reduced the SSD amplitude and increased the AP threshold ($p < 0.05$). The threshold for Ca^{2+} -transients (1.40 ± 0.04 kV/cm) was not significantly affected by a tetrodotoxin-verapamil cocktail, suggesting the activation of a Ca^{2+} entry pathway independent from opening of Na^+ or Ca^{2+} voltage-gated channels. Removal of external Ca^{2+} decreased the SSD amplitude ($p = 0.004$) and blocked Ca^{2+} -transients but not APs. The incidence of transient afterdepolarization waves was decreased by verapamil and by removal of external Ca^{2+} ($p = 0.002$).

Conclusions.—The study established that nsPEF stimulation caused calcium entry into cardiac myocytes (including routes other than voltage-gated calcium channels) and SSD. Tetrodotoxin-sensitive APs were mediated by SSD, whose amplitude depended on the calcium entry. Plasma membrane electroporation was the most likely primary mechanism of SSD with additional contribution from L-type calcium and sodium-calcium exchanger currents.

*Correspondence: Andrei G. Pakhomov, PhD, Frank Reidy Research Center for Bioelectronics, Old Dominion University, 4211 Monarch Way, Ste. 300, Norfolk, Virginia, USA, 23508. Phone: +1(210)2049012, +1(757)6838003, Fax: +1(757)4511010. apakhomo@odu.edu.

Disclosures: None

Keywords

action potential; cardiac myocytes; electroporation; ion channels; nsPEF

1. INTRODUCTION

Defibrillation is a recognized life-saving procedure used in conditions of cardiac arrest. Insufficient electric field strength, tissue injury and reinduction of fibrillation can decrease the effectiveness of defibrillation¹. Since the extent of tissue injury depends on the duration of shocks^{2, 3}, nanosecond pulsed electric field (nsPEF) stimulation has been recently proposed as a novel defibrillation modality to achieve higher efficiency of defibrillation on the first shock along with a profound reduction in the shock energy, minimized side effects, and low probability of reinduction of arrhythmias⁴. The mechanism of nsPEF-related defibrillation is unclear, but it most likely implies that nsPEF induces myocardial excitation that fills in the excitable gap ahead of reentrant activation wave and terminates reentry thereby. The potentially beneficial features of nsPEF defibrillation are linked to the involvement of displacement currents in membrane charging^{5–8}, which reduces the needed shock energy, and the excitation is achieved simultaneously and more uniformly under the anode and the cathode and in the volume between them. Charging of cell membranes by nsPEF is less affected by tissue inhomogeneities^{6, 9} such as post-infarction scars, thereby lowering the chance of reentry arrhythmias and re-fibrillation. In case of electroporative damage to cardiomyocytes by intense electric field, nsPEF-opened membrane pores are limited to 1-1.5 nm diameter (“nanoelectropores”)^{10–13}, so the transmembrane leaks are less damaging than after conventional defibrillation with millisecond pulses. Finally, transient inhibition of voltage-gated Na⁺ and Ca²⁺ channels^{14, 15} may assist the antiarrhythmic effect of nsPEF defibrillation.

Recent studies confirmed nsPEF efficiency for both the defibrillation in Langendorff-perfused rabbit hearts⁶ and the reduction of propidium uptake (a standard marker for electroporative damage) in individual cardiomyocytes exposed to electric shocks proportionally higher than the stimulation threshold^{16, 17}. At the same time, repeated 200- or 800-ns shocks just above the stimulation threshold triggered abnormal Ca²⁺ responses manifested as prolonged elevations of the resting Ca²⁺ level, Ca²⁺ waves, or distorted Ca²⁺ transients¹⁶. These abnormalities were thought to be of potential concern for nsPEF use in defibrillation. The underlying mechanisms have not been explored yet and may involve permeabilization of the sarcoplasmic reticulum by nsPEF^{18–20}, damage to voltage-gated Ca²⁺ channels¹⁵, alteration of phosphoinositide signaling^{21–23}, and nanoelectroporation of the cell membrane (which can be detected by the loss of membrane potential and transport of water and small ions, but not by propidium uptake^{11, 12, 19, 24–26}). The present study was aimed at testing the latter mechanism by exploring if there are any abnormal responses upstream from Ca²⁺ handling, namely at the level of the action potential (AP) induction by nsPEF

Indeed, the mechanism of generation of nsPEF-induced APs remains poorly understood. Such stimuli may be too brief to elicit APs directly by membrane depolarization, since the

process of opening of voltage-gated sodium channels, as evidenced by the time course of gating currents, takes tens to hundreds of microseconds^{27, 28}. Although gating at the single molecule level might be a “jump process” between discrete states^{28, 29}, with probability increased by depolarization, nsPEF-induced depolarization may be too brief to open the critical number of channels to trigger an AP. It suggests that there might be a coupling mechanism to link nsPEF and AP generation. For conventional (long) stimuli, it was demonstrated that electroporation caused by a strong shock leads to a prolonged depolarization, which can result in excitation of cardiac muscle³⁰. In a similar manner, electroporation may be involved in nsPEF-induced AP generation. Experimental findings suggest that some electroporative damage can already take place at nsPEF threshold for cell excitation^{25, 31, 32} (with the exception of peripheral nerve stimulation^{33, 34}), although the causal connection has not been proven and remains questionable²⁵.

The objective of the present study was to evaluate the mechanism of AP induction by nsPEF in murine cardiac myocytes, and specifically to assess the role of nanoelectroporation in myocyte excitation. At first, we compared APs evoked by conventional and nsPEF stimulation. Then, we assessed the role of inward ionic currents in the generation of nsPEF-induced APs using ion channel blockers. Finally, to evaluate the relationship between cell excitation and nanoelectroporation, we analyzed cytosolic calcium responses to nsPEF stimuli.

2. Methods

2.1. Myocyte isolation

Experiments were performed in enzymatically isolated ventricular myocytes from 14 DBA/2J murine hearts. The animal handling protocol conformed to the *Guide for the Care and Use of Laboratory Animals, 8th Edition* published by the National Academies Press (US) 2011, and was approved by the Institutional Animal Care and Use Committee (IACUC protocol #16-016, May 2, 2016). The procedure of cell isolation was described elsewhere³⁵. Briefly, mice were injected with heparin (50 IU ip), anesthetized with isoflurane, and sacrificed by cervical dislocation. Heart was quickly excised and aorta was cannulated. Heart was first perfused with a perfusion buffer containing (in mM): 113 NaCl, 4.7 KCl, 1.2 MgSO₄, 0.6 Na₂HPO₄, 0.6 KH₂PO₄, 12 NaHCO₃, 10 KHCO₃, 30 Taurine, 5.5 Glucose, 10 2,3-Butanedione monoxime, and 10 HEPES (pH 7.4) (all chemicals from Sigma-Aldrich, St. Louis, MO, USA). In 5 min, perfusion was switched to a digestion buffer [perfusion buffer supplemented with 0.1 mg/ml Liberase TM (Roche, Switzerland)]. After 7 min of perfusion with the digestion buffer at 37°C, the ventricles were minced, the cell suspension was filtered, and digestion was stopped by adding bovine serum albumin. Next, cells were placed in a control buffer containing (in mM): 133.5 NaCl, 4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 11 Glucose, 10 HEPES; and 0.1% BSA (pH 7.4), supplemented with 200 µM CaCl₂. CaCl₂ was then added in a stepwise manner to a target concentration of 1 mM (all chemicals from Sigma-Aldrich, St. Louis, MO, USA). The cells were allowed to settle down on laminin-coated glass cover slips. The final control buffer solution was supplemented with 1% of 100x penicillin/streptomycin (Corning, Corning, NY, USA), and 1% of 100X insulin-

transferrin-selenium (Gibco, Gaithersburg, MD, USA). Cells were kept at the room temperature and used in experiments within 24 hours.

2.2. Cell Imaging

Optical recordings of the membrane potential were accomplished with FluoVolt dye (Thermo Fisher Scientific, Waltham, MA, USA), which was previously validated for the recording of neuronal APs in our group²⁵ and for cardiac APs by others³⁶. Cells were loaded with FluoVolt in Tyrode solution composed of (in mM): 140 NaCl, 5.4 KCl, 1 CaCl₂, 1.5 MgCl₂, 10 HEPES, 10 glucose (pH 7.3) (all chemicals from Sigma-Aldrich, St. Louis, MO, USA), supplemented with the dye (1:1000×) and PowerLoad Concentrate (1:100×) (Thermo Fisher Scientific, Waltham, MA, USA), for 5 minutes. Imaging of cytosolic Ca²⁺ dynamics was performed with Fluo-4 dye (Invitrogen, Carlsbad, CA, USA) loaded in the cells by a 30-minute incubation in the Tyrode solution containing 5 μM of Fluo-4 AM. Both dyes were loaded at room temperature in the dark.

After dye loading, coverslips with cells were placed in a glass-bottomed perfusion chamber (Warner Instruments, Hamden, CT, USA) mounted on a stage of an IX71 microscope (Olympus America, Center Valley, PA, USA) and imaged with a PlanApo N 60, 1.42 NA objective (Olympus). Time-lapse image stacks (3,000 frames/s for 200 ms) were acquired with an iXon Ultra 897 back-illuminated CCD Camera (Andor Technology, Belfast, UK) using Solis interface (Andor). The camera sensor outside the area of interest was physically shielded with an Optomask (Andor). In separate experiments, images were acquired at 2 frames/s for 1 minute. The dyes were excited with a Sola SE diode light source (Excelitas Technologies Corp., Waltham, MA, USA) using a standard FITC filter cube. The light source, the camera, and the nsPEF generator (or a Grass stimulator for longer pulses) were controlled by a TTL pulse protocol using Digidata 1440A board and Clampex v. 10.2 software (Molecular Devices, Sunnyvale, CA, USA).

To measure the time-course of fluorescence change due to bleaching, the first stack of images in each cell was acquired without nsPEF stimulation (sham exposure). Then the cell was stimulated by 200-ns trapezoidal PEF. nsPEF stimuli were applied with 10% stepwise amplitude increments to determine the threshold for AP, slow depolarization, or calcium mobilization. In a separate set of experiments, we compared AP amplitude and duration for conventional and nsPEF excitation, and each cell (n=12) was stimulated consecutively by 200-μs and 200-ns pulses at 30% above the threshold. Stimuli were delivered at 50 ms or 10 s into the 200-ms and 1-minute image acquisitions, respectively.

The procedures for field stimulation of individual selected cells on a microscope stage were similar to those described previously²⁵. In brief, a single selected cell was brought in the center of the microscope field of view, and a pair of tungsten rod electrodes (100 μm diameter, 170 μm inter-electrode gap) was positioned above it with a help of a micromanipulator. The cell was in the middle of the gap between the electrodes, with its long axis aligned with the electrodes (+/- 30°). The tips of the electrodes were placed at precisely 50 μm above the cover slip surface. Conventional stimuli (200 μs width) were delivered from a Grass S88 stimulator (Grass Instrument, Quincy, MA, USA). nsPEF stimuli were produced by a custom-made MOSFET-based generator controlled by Model 577

digital delay generator (Berkley Nucleonics Corp., San Rafael, CA, USA). The pulse shape and amplitude were monitored by a model 5204 Oscilloscope (Tektronix, Inc., Beaverton, OR, USA). Electric field strength at a cell location were calculated using 3D numerical simulations using a commercial finite element solver COMSOL Multiphysics, Release 5.0 (COMSOL Inc., Stockholm, Sweden); see the previous report²¹ for details.

We applied tetrodotoxin (TTX, 50 μ M, Alomone Labs, Jerusalem, Israel), verapamil (20 μ M, Ascent Scientific, Bristol, UK), SEA0400 (1 μ M, Sigma-Aldrich, St. Louis, MO, USA) and Ca-free Tyrode solution for modification of voltage-gated sodium channel-dependent current (I_{Na}), L-type voltage-gated calcium channel-dependent current (I_{Ca-L}), current of sodium-calcium exchanger (I_{NCX}) and exclusion of external calcium influx, respectively. TTX, verapamil and SEA0400 were added to normal Tyrode solution in the perfusion chamber. Ca-free Tyrode solutions replaced normal Tyrode during dye loading and in the perfusion chamber.

2.3. Data processing

Membrane potential changes were calculated off-line. Fluorescence signal taken during sham exposures was fitted with a 4th degree polynomial function. Both the fitted data (bleaching) and the data from experiments with nsPEF stimulation were normalized to the baseline (the average fluorescence during 10 ms immediately before the pulse), and bleaching was subtracted from experimental data. The slow sustained depolarization (SSD) amplitude was measured as average fluorescence increase in the interval from 50 to 130 ms after pulse application in respect to the baseline level. APs, if present in this time interval, were manually cut out and excluded from averaging. Peak latency of APs was measured as the interval between pulse application and AP peak. AP amplitude was measured as the difference between the baseline level and the maximal value of fluorescence during AP. AP duration was measured at 50% of AP height.

Statistical analysis was performed with SPSS 24 (SPSS, Inc., Chicago, Illinois, USA). Parametrical paired and nonpaired Student's t-tests were applied to Gaussian data distributions, as judged by Kolmogorov-Smirnov test. One-way ANOVA was used for multiple comparisons (Dunnet test). The data are presented as mean $\pm$ SEM, if not indicated otherwise. Linear regression analysis was performed to evaluate associations between different AP parameters. The incidence of transient depolarization waves in different solutions was compared by the chi-square test. The differences were considered significant at $p < 0.05$.

3. RESULTS

Individual cells which could be excited by 200 μ s stimuli (i.e., proven viable and excitable) could also be excited with 200-ns PEF. APs elicited by suprathreshold nsPEF did not differ from those evoked by conventional pulses in amplitude ($13.83 \pm 2.12\%$ vs $14.25 \pm 2.49\%$, respectively, $p=0.558$) or duration (5.71 ± 0.80 vs 5.33 ± 0.76 ms, respectively, $p=0.378$, Fig. 1A). However, cell responses to nsPEF were different in several aspects. At suprathreshold stimulus amplitudes, nsPEF-induced APs could arise with a minimal or no measurable delay but were always followed by SSD after AP (Fig. 1A). Increasing nsPEF amplitude reduced

the peak latency and increased the SSD amplitude (Fig. 1B). The SSD response lasted tens of seconds (Fig. 1C), suggesting the involvement of mechanisms other than physiological opening of voltage-gated ion channels. APs in approximately 2/3 of cells were followed by transient afterdepolarization waves superimposed on SSD (Fig. 1A and B; Table 1) and sometimes giving rise to additional APs. While transient afterdepolarizations were occasionally observed with conventional stimulation as well, their incidence was much lower (Table 1).

At the threshold amplitude, nsPEF induced APs with a significant delay and SSD always preceded APs (Fig. 2). SSD duration in each individual cell depended on its excitability, and myocytes having a higher AP threshold had a longer AP latency. The peak latency of AP (as measured at stimulation threshold) ranged from 1.3 to as much as 132 ms and was significantly associated with the threshold nsPEF strength (univariate linear regression analysis, $B=0.851$, 95% CI 0.482 – 1.220, $p<0.001$). Out of a total of 85 cells that were excited by nsPEF, the peak latency at threshold nsPEF stimulation exceeded 10, 20, and 50 ms in 60, 43, and 17 cells, respectively. The electric field threshold for SSD was lower than for AP (1.26 ± 0.03 vs 1.34 ± 0.03 kV/cm, respectively; mean difference 0.08 ± 0.01 kV/cm with paired t-test, $p<0.001$).

In contrast to conventional pulses, which could evoke APs many times, nsPEF stimulation often damaged cells. However, Fig. 2 shows that nsPEF stimuli could induce APs repeatedly, but only at the threshold stimulus intensity. Once nsPEF amplitude was increased to elicit strong SSD, the next stimulation usually failed to evoke an AP (data not shown).

We then attempted to identify the major inward current responsible for AP generation by nsPEF stimulation. With rare exceptions (in 21 out of 25 cells), TTX application consistently blocked AP generation (Fig. 3). Addition of verapamil to Tyrode solution decreased the incidence of transient afterdepolarization waves (Table 1, $p=0.002$) but did not block the generation of AP (Fig. 3). These data suggest that nsPEF stimulation evoked typical sodium current-dependent APs.

SSD was not eliminated by either the inhibition of sodium and L-type calcium currents with TTX and verapamil, respectively, or by the removal of Ca^{2+} from the bath solution (Fig. 3). However, the amplitude of SSD was lower in the conditions that impaired calcium influx (verapamil, Ca^{2+} -free Tyrode solution, Fig. 4A). TTX decreased SSD amplitude as well, but this effect did not reach the level of statistical significance, which might be related to the insufficient statistical power in case of multiple comparisons. AP threshold was higher in cells treated with verapamil (Fig. 4B).

Collectively, these data suggested that calcium transport (including transport via ion channels) was involved in the SSD development but did not solely account for it. SSD could be caused by the electroporation of either plasma or sarcoplasmic reticulum (SR) membranes. Electroporation of the plasma membrane would allow extracellular cations to enter the cell causing the loss of the resting membrane potential. SR electroporation would cause a leak of Ca^{2+} and its accumulation in the sarcoplasm. If this is the case, Ca^{2+} ions are

expected to be extruded from the cell by the forward mode of 3:1 sodium-calcium exchange (I_{NCX}), resulting in depolarization.

This latter mechanism was tested by inhibition of I_{NCX} . Application of a specific I_{NCX} blocker SEA0400 did not preclude AP generation (Fig. 3). However, it decreased the SSD amplitude (Fig. 3 and Fig. 4A) and increased the AP threshold (Fig. 4B). This means that whatever the source of intracellular calcium was (influx of external Ca^{2+} ions or a leak from SR), the sodium-calcium exchanger contributed to SSD development but was not critical for AP generation by nsPEF.

Next, we analyzed nsPEF-induced Ca^{2+} transients in order to elucidate the mechanism of I_{NCX} involvement. nsPEF stimulation evoked Ca^{2+} transients at the threshold of 1.40 ± 0.04 kV/cm (Fig. 5A), the intensity similar to the AP generation threshold (Fig. 4B). In the presence of TTX and verapamil, a combination preventing generation of AP and calcium channel opening, we still observed a distinct rise of intracellular calcium concentration, though relatively slow (Fig. 5B). The threshold for calcium response in the presence of TTX and verapamil (1.46 ± 0.07 kV/cm) did not differ from either the threshold in normal Tyrode or the AP generation threshold ($p > 0.05$). These observations indicated that calcium was coming either from SR or from outside but not through voltage-gated calcium channels. In order to confirm or exclude the role of SR as a source of calcium, we performed similar recordings in cells kept in a Ca-free Tyrode solution (Fig. 5C). An observation of a calcium response in the Ca-free bath medium would prove that Ca^{2+} ions come from SR. However, we observed no Ca^{2+} -transients in Ca-free solutions within the studied range of nsPEF strength (1.14-3.30 kV/cm), which means that external Ca^{2+} was needed for triggering calcium release from SR and that there was no SR electroporation in the studied conditions.

4. DISCUSSION

In the present study, we demonstrated that nsPEF stimulation induced I_{Na} -dependent AP. In contrast to the previous study³⁷, we showed the role of I_{Na} in AP generation by the TTX-dependent block. While APs evoked by nanosecond and microsecond stimuli were similar in amplitude and duration, the SSD phenomenon causing long delays in AP generation and afterdepolarization appeared to be a signature of nsPEF-induced APs. In a stark contrast to conventional stimulation, short peak latencies in nsPEF-induced APs were strongly associated with high SSD amplitude measured after APs (Fig. 1B).

The SSD and AP thresholds were close to each other with the SSD threshold being lower than that of AP. In addition, cells with higher AP threshold showed slower SSD rise and more delayed AP onset. This relationship suggests that it was SSD that caused AP after nsPEF, at least at the threshold level for AP induction. However, APs at suprathreshold stimulation intensities might start instantaneously after the stimulus (Fig. 1). It is not clear if the shorter latency was a result of stronger SSD (which would bring the membrane to the AP threshold faster) or were directly caused by nsPEF, similarly to the traditional electrostimulation with micro- and millisecond pulses. An incontrovertible distinction between these two mechanisms would require an observation of an AP elicited by nsPEF in the absence of SSD. We have been specifically looking for such an event, by testing various

stimulation protocols and drug treatments, but have not observed it. We conclude that SSD always accompanied nsPEF-induced APs and was the likely cause of it near the AP threshold, although the causal connection for more intense stimuli remains in question. Of note, studies of the interplay of electroporabilization and excitation by nsPEF in cultured neurons arrived to a similar conclusion³⁴.

SSD may be considered a result of several mechanisms, which do not exclude each other. Among them are opening of selective and/or nonselective cation channels, forward mode of sodium-calcium exchanger, and electroporation. The persistence of SSD when major inward currents are inhibited (I_{Na} by TTX and I_{Ca-L} by verapamil or removal of external Ca^{2+}) implies that opening of voltage-gated sodium and calcium channels was not required for SSD. Nevertheless, the decrease of SSD amplitude by verapamil and external Ca^{2+} removal suggested the involvement of some calcium-dependent mechanism in SSD development.

Such a mechanism could be the forward mode of the sodium-calcium exchanger. This transporter extrudes 1 Ca^{2+} from the cell in exchange for 3 Na^{+} , resulting in depolarization. Indeed, the inhibition of I_{NCX} with SEA0400 led to the increase of AP threshold and decrease of SSD amplitude. However, neither SSD nor AP generation were prevented by SEA0400. Consistently with the data on the effects of verapamil and Ca^{2+} removal, the effect of SEA0400 implies that I_{NCX} served as a supporting mechanism for SSD. Another candidate for calcium-dependent depolarization can be one of nonselective cation channels like TRPM4 demonstrating voltage and calcium sensitivity^{38, 39}.

Accumulation of calcium might be caused by either the influx of ions from the outside or by SR leakage due to its electroporation, as reported earlier^{19, 20, 40}. However, the absence of Ca^{2+} transients in Ca-free solutions demonstrates that SR was not electroporated by the nsPEF, at least in the studied range of nsPEF strengths. It also suggested that cytosolic calcium accumulation was not necessary for SSD or AP generation since both were observed in Ca-free solutions.

Taken together with the above, the presence of calcium responses when calcium channel opening was prevented by the combination of TTX and verapamil indicates that calcium entered the cell from the external medium but not via voltage-gated channels. It could be due to either plasma membrane electroporation or opening of nonselective channels, like TRPC3, TRPC6, or TRPM4 associated with electrophysiological effects in myocardium^{41, 42}. However, the membrane potential changes which last tens of seconds (Fig. 1C) evidence in favor of electroporation. The long time course of nsPEF-induced SSD can account for the progressive distortion of calcium transients under repetitive stimulation with 200 ns stimuli observed in the recent study⁴³.

Both the time course and drug sensitivity profile of SSD point to cell membrane nanoelectroporation by nsPEF as the most likely underlying mechanism. Cell membrane permeabilized by nsPEF develops permeability to small inorganic cations, resulting in increased electric conductivity and depolarization, while maintaining little or no permeability to larger solutes such as propidium cation^{10, 12, 44–46}. However, several observations made in the present study imply the involvement of active mechanism(s) in the

development of SSD in addition to the electroporation. I_{NCX} and I_{Ca-L} , though not critical for either SSD development or AP firing, were shown to affect both. Blockers of these currents decreased the amplitude of SSD, thereby increasing the AP threshold.

Afterdepolarizations (categorized as early and delayed ones) are important electrophysiological phenomena closely related to myocyte calcium handling and arrhythmogenesis, and usually ascribed to (re)activation of I_{Ca-L} or I_{NCX} , respectively^{47–49}. Though the short duration and triangular morphology of murine ventricular AP make strict categorization difficult, transient afterdepolarization waves seen in our study under nsPEF stimulation could be technically referred to as the delayed afterdepolarizations. However, our results suggest that the mechanism of these transient afterdepolarization waves is related to the L-type calcium channels, which are thought to be involved in generation of the early afterdepolarizations^{47–49}. The incidence of these waves was reduced by interventions targeting I_{Ca-L} , (i.e., verapamil and removal of calcium ions from the extracellular medium) and was not changed by I_{NCX} inhibition with SEA0400 (Table 1).

The fact that nsPEF stimulation can consistently induce AP generation can be considered promising for further studies of nsPEF as a potential defibrillation modality. However, findings of the present study demonstrate that AP generation in cardiomyocytes was primarily due to electroporation, which has been also found in hearts undergoing conventional electric shocks of defibrillation intensity^{50, 51}. The presence of electroporation usually raises concerns related to its probable complications, like cell death or arrhythmia. Considering the context of ventricular fibrillation treatment, it is noteworthy that electroporation-induced SSD accompanying APs can modify cellular excitability in either direction. Appearance of the transient afterdepolarization waves after nsPEF stimuli observed in the present study reflects the increase of excitability and may impose additional arrhythmia risks. On the other hand, it has been pointed out that electroporation can confer either antiarrhythmic or proarrhythmic properties on myocardium, both may result from the suppression of excitability after AP^{30, 52, 53}. These changes may provoke unpredictable thus far electrophysiological consequences in clinical settings.

5. CONCLUSION

This study demonstrated that 200-ns stimuli induced APs in enzymatically isolated adult murine ventricular cardiomyocytes. nsPEF caused Ca^{2+} entry, associated with an SSD, which eventually culminated in an AP. This Ca^{2+} entry could bypass voltage-gated calcium channels, although the sodium-calcium exchanger and L-type calcium currents both contributed to the SSD. We conclude that the electroporative damage of the cell membrane was the most likely primary mechanism of excitation of cardiomyocytes by 200-ns nsPEF, at least at the near-threshold stimulus intensities.

Acknowledgments

Funding: This work was supported by the National Heart, Lung, and Blood Institute at the National Institutes of Health [R01HL128381 to A.G.P.]; and AFOSR MURI grant on Nanoelectropulse-induced Electromechanical Signaling and Control of Biological Systems, administered through Old Dominion University [FA9550-15-1-0517 to A.G.P.]

REFERENCES

1. Dosdall DJ, Fast VG and Ideker RE. Mechanisms of defibrillation. Annual review of biomedical engineering. 2010;12:233–58.
2. Prado LN, Goulart JT, Zoccoler M and Oliveira PX. Ventricular myocyte injury by high-intensity electric field: Effect of pulse duration. General physiology and biophysics. 2016;35:121–30. [PubMed: 26830130]
3. Nichol G, Sayre MR, Guerra F and Poole J. Defibrillation for Ventricular Fibrillation: A Shocking Update. Journal of the American College of Cardiology. 2017;70:1496–1509. [PubMed: 28911514]
4. Varghese F, Neuber JU, Xie F, Philpott JM, Pakhomov AG and Zemlin CW. Low-energy defibrillation with nanosecond electric shocks. Cardiovasc Res. 2017;113:1789–1797. [PubMed: 29016714]
5. Pakhomov AG, Miklavcic D and Markov MS. Advanced Electroporation Techniques in Biology in Medicine. 2010:528
6. Varghese F, Neuber JU, Xie F, Philpott JM, Pakhomov AG and Zemlin CW. Low-Energy Defibrillation with Nanosecond Electric Shocks. Cardiovasc Res. 2017.
7. Xie F, Varghese F, Pakhomov AG, Semenov I, Xiao S, Philpott J and Zemlin C. Ablation of Myocardial Tissue With Nanosecond Pulsed Electric Fields. PLoS One. 2015;10:e0144833. [PubMed: 26658139]
8. Kotnik T and Miklavcic D. Second-order model of membrane electric field induced by alternating external electric fields. Ieee T Bio-Med Eng. 2000;47:1074–1081.
9. Gowrishankar TR and Weaver JC. An approach to electrical modeling of single and multiple cells. P Natl Acad Sci USA. 2003;100:3203–3208.
10. Pakhomov AG, Gianulis E, Vernier PT, Semenov I, Xiao S and Pakhomova ON. Multiple nanosecond electric pulses increase the number but not the size of long-lived nanopores in the cell membrane. Biochim Biophys Acta. 2015;1848:958–66. [PubMed: 25585279]
11. Nesin OM, Pakhomova ON, Xiao S and Pakhomov AG. Manipulation of cell volume and membrane pore comparison following single cell permeabilization with 60- and 600-ns electric pulses. Biochim Biophys Acta. 2011;1808:792–801. [PubMed: 21182825]
12. Pakhomov AG and Pakhomova ON. Nanopores: A distinct transmembrane passageway in electroporated cells In: Pakhomov AG, Miklavcic D and Markov MS, eds. *Advanced Electroporation Techniques in Biology in Medicine* Boca Raton: CRC Press; 2010: 178–194.
13. Pakhomov AG, Bowman AM, Ibey BL, Andre FM, Pakhomova ON and Schoenbach KH. Lipid nanopores can form a stable, ion channel-like conduction pathway in cell membrane. Biochem Biophys Res Commun. 2009;385:181–6. [PubMed: 19450553]
14. Nesin V and Pakhomov AG. Inhibition of voltage-gated Na(+) current by nanosecond pulsed electric field (nsPEF) is not mediated by Na(+) influx or Ca(2+) signaling. Bioelectromagnetics. 2012;33:443–51. [PubMed: 22234846]
15. Nesin V, Bowman AM, Xiao S and Pakhomov AG. Cell permeabilization and inhibition of voltage-gated Ca(2+) and Na(+) channel currents by nanosecond pulsed electric field. Bioelectromagnetics. 2012;33:394–404. [PubMed: 22213081]
16. Semenov I, Grigoryev S, Neuber JU, Zemlin CW, Pakhomova ON, Casciola M and Pakhomov AG. Excitation and injury of adult ventricular cardiomyocytes by nano- to millisecond electric shocks. Sci Rep. 2018;8:8233. [PubMed: 29844431]
17. Semenov I, Zemlin C, Pakhomova ON, Xiao S and Pakhomov AG. Diffuse, non-polar electropermeabilization and reduced propidium uptake distinguish the effect of nanosecond electric pulses. Biochim Biophys Acta. 2015;1848:2118–25. [PubMed: 26112464]
18. Semenov I, Casciola M, Ibey BL, Xiao S and Pakhomov AG. Electropermeabilization of cells by closely spaced paired nanosecond-range pulses. Bioelectrochemistry. 2018;121:135–141. [PubMed: 29413863]
19. Semenov I, Xiao S and Pakhomov AG. Primary pathways of intracellular Ca(2+) mobilization by nanosecond pulsed electric field. Biochim Biophys Acta. 2013;1828:981–9. [PubMed: 23220180]

20. Semenov I, Xiao S, Pakhomova ON and Pakhomov AG. Recruitment of the intracellular Ca²⁺ by ultrashort electric stimuli: the impact of pulse duration. *Cell Calcium*. 2013;54:145–50. [PubMed: 23777980]
21. Tolstykh GP, Beier HT, Roth CC, Thompson GL and Ibey BL. 600 ns pulse electric field-induced phosphatidylinositol4,5-bisphosphate depletion. *Bioelectrochemistry*. 2014;100:80–7. [PubMed: 24530104]
22. Tolstykh GP, Beier HT, Roth CC, Thompson GL, Payne JA, Kuipers MA and Ibey BL. Activation of intracellular phosphoinositide signaling after a single 600 nanosecond electric pulse. *Bioelectrochemistry*. 2013;94:23–9. [PubMed: 23747521]
23. Tolstykh GP, Tarango M, Roth CC and Ibey BL. Nanosecond pulsed electric field induced dose dependent phosphatidylinositol-4,5-bisphosphate signaling and intracellular electro-sensitization. *Biochim Biophys Acta*. 2017;1859:438–445.
24. Bowman AM, Nesin OM, Pakhomova ON and Pakhomov AG. Analysis of plasma membrane integrity by fluorescent detection of Tl(+) uptake. *J Membr Biol*. 2010;236:15–26. [PubMed: 20623351]
25. Casciola M, Xiao S and Pakhomov AG. Damage-free peripheral nerve stimulation by 12-ns pulsed electric field. *Sci Rep*. 2017;7:10453. [PubMed: 28874684]
26. Vernier PT, Sun Y, Marcu L, Salem S, Craft CM and Gundersen MA. Calcium bursts induced by nanosecond electric pulses. *Biochemical and Biophysical Research Communications*. 2003;310:286–295. [PubMed: 14521908]
27. Hille B *Ionic channels of excitable membranes*. 2nd ed. Sunderland, Massachusetts: Sinauer Associates, Inc.; 1992.
28. Bezanilla F Gating currents. *J Gen Physiol*. 2018;150:911–932. [PubMed: 29941430]
29. Conti F and Stuhmer W. Quantal charge redistributions accompanying the structural transitions of sodium channels. *Eur Biophys J*. 1989;17:53–9. [PubMed: 2548829]
30. Nikolski VP and Efimov IR. Electroporation of the heart. *Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology*. 2005;7 Suppl 2:146–54.
31. Craviso GL, Choe S, Chatterjee P, Chatterjee I and Vernier PT. Nanosecond electric pulses: a novel stimulus for triggering Ca²⁺ influx into chromaffin cells via voltage-gated Ca²⁺ channels. *Cell Mol Neurobiol*. 2010;30:1259–65. [PubMed: 21080060]
32. Wang S, Chen J, Chen MT, Vernier PT, Gundersen MA and Valderrabano M. Cardiac myocyte excitation by ultrashort high-field pulses. *Biophysical journal*. 2009;96:1640–8. [PubMed: 19217879]
33. Lamberti P, Compitiello M, Romeo S, Lamberti P, Compitiello M, Romeo S, Lamberti P, Romeo S and Compitiello M. ns Pulsed Electric Field-Induced Action Potentials in the Circuitual Model of an Axon. *IEEE transactions on nanobioscience*. 2018;17:110–116. [PubMed: 29870334]
34. Pakhomov AG, Semenov I, Casciola M and Xiao S. Neuronal excitation and permeabilization by 200-ns pulsed electric field: An optical membrane potential study with FluoVolt dye. *Biochim Biophys Acta*. 2017;1859:1273–1281.
35. Pakhomov AG, Grigoryev S, Semenov I, Casciola M, Jiang C and Xiao S. The second phase of bipolar, nanosecond-range electric pulses determines the electroporation efficiency. *Bioelectrochemistry (Amsterdam, Netherlands)*. 2018;122:123–133.
36. McPheeters MT, Wang YT, Werdich AA, Jenkins MW and Laurita KR. An infrared optical pacing system for screening cardiac electrophysiology in human cardiomyocytes. *PloS one*. 2017;12:e0183761. [PubMed: 28837652]
37. Wang S, Chen J, Chen MT, Vernier PT, Gundersen MA and Valderrabano M. Cardiac myocyte excitation by ultrashort high-field pulses. *Biophysical journal*. 2009;96:1640–8. [PubMed: 19217879]
38. Simard C, Salle L, Rouet R and Guinamard R. Transient receptor potential melastatin 4 inhibitor 9-phenanthrol abolishes arrhythmias induced by hypoxia and re-oxygenation in mouse ventricle. *British journal of pharmacology*. 2012;165:2354–64. [PubMed: 22014185]

39. Hu Y, Duan Y, Takeuchi A, Hai-Kurahara L, Ichikawa J, Hiraishi K, Numata T, Ohara H, Iribe G, Nakaya M, Mori MX, Matsuoka S, Ma G and Inoue R. Uncovering the arrhythmogenic potential of TRPM4 activation in atrial-derived HL-1 cells using novel recording and numerical approaches. *Cardiovascular research*. 2017;113:1243–1255. [PubMed: 28898995]
40. Schoenbach KH, Hargrave SJ, Joshi RP, Kolb JF, Nuccitelli R, Osgood C, Pakhomov A, Stacey M, Swanson RJ, White JA, Xiao S, Zhang J, Beebe SJ, Blackmore PF and Buescher ES. Bioelectric Effects of Intense Nanosecond Pulses. *IEEE Transactions on Dielectrics and Electrical Insulation*. 2007;14:1088–1109.
41. Yue Z, Xie J, Yu AS, Stock J, Du J and Yue L. Role of TRP channels in the cardiovascular system. *American Journal of Physiology-Heart and Circulatory Physiology*. 2014;308:H157–H182. [PubMed: 25416190]
42. Guinamard R, Bouvagnet P, Hof T, Liu H, Simard C and Salle L. TRPM4 in cardiac electrical activity. *Cardiovascular research*. 2015;108:21–30. [PubMed: 26272755]
43. Semenov IG S; Neuber J; Zemlin CW; Pakhomova ON; Casciola M; Pakhomov AG Excitation and injury of adult ventricular cardiomyocytes by nano- to millisecond electric shocks. *Scientific Reports*. 2018.
44. Schoenbach KH. Bioelectric effect of intense nanosecond pulses In: Pakhomov, D. AGM; Markov MS, ed. *Advanced electroporation techniques in biology and medicine* Boca Raton, Florida, USA: CRC Press Taylor and Francis Group; 2010: 19–49.
45. Pakhomov AG, Kolb JF, White JA, Joshi RP, Xiao S and Schoenbach KH. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). *Bioelectromagnetics*. 2007;28:655–63. [PubMed: 17654532]
46. Ibey BL, Xiao S, Schoenbach KH, Murphy MR and Pakhomov AG. Plasma membrane permeabilization by 60- and 600-ns electric pulses is determined by the absorbed dose. *Bioelectromagnetics*. 2009;30:92–99. [PubMed: 18839412]
47. Sipido KR, Bito V, Antoons G, Volders PG and Vos MA. Na/Ca exchange and cardiac ventricular arrhythmias. *Annals of the New York Academy of Sciences*. 2007;1099:339–48. [PubMed: 17446474]
48. Benitah J- P, Alvarez JL and Gómez AM. L-type Ca²⁺ current in ventricular cardiomyocytes. *Journal of molecular and cellular cardiology*. 2010;48:26–36. [PubMed: 19660468]
49. Landstrom AP, Dobrev D and Wehrens XHT. Calcium Signaling and Cardiac Arrhythmias. *Circulation research*. 2017;120:1969. [PubMed: 28596175]
50. Nikolski VP, Sambelashvili AT, Krinsky VI and Efimov IR. Effects of electroporation on optically recorded transmembrane potential responses to high-intensity electrical shocks. *American journal of physiology Heart and circulatory physiology*. 2004;286:H412–8. [PubMed: 14527941]
51. Wang YT, Efimov IR and Cheng Y. Electroporation induced by internal defibrillation shock with and without recovery in intact rabbit hearts. *American journal of physiology Heart and circulatory physiology*. 2012;303:H439–49. [PubMed: 22730387]
52. Al-Khadra A, Nikolski V and Efimov IR. The role of electroporation in defibrillation. *Circulation research*. 2000;87:797–804. [PubMed: 11055984]
53. Ashihara T, Yao T, Namba T, Ito M, Ikeda T, Kawase A, Toda S, Suzuki T, Inagaki M, Sugimachi M, Kinoshita M and Nakazawa K. Electroporation in a model of cardiac defibrillation. *Journal of cardiovascular electrophysiology*. 2001;12:1393–403. [PubMed: 11797997]

Figure 1.

Representative recordings of membrane potentials under nsPEF and conventional stimulation (vertical dotted red lines indicate the time of pulse application). A) Superposition of two consecutive recordings done in the same cell under stimulation with first 200 μ s (blue) and then 200 ns (red) duration pulses of suprathreshold amplitude (0.174 and 2.55 kV/cm, respectively). AP amplitude and AP duration on 50% repolarization level were nearly the same in the two conditions. However, SSD with additional transient afterdepolarization waves (black arrows) were typically observed in nsPEF stimulation. B)

Changes of membrane potential response to 200 ns stimuli with increasing the pulse voltage in the same cell. With the minimal nsPEF strength (red), only SSD response was observed. The just-above-threshold stimulus (orange) elicited an AP with the maximal peak latency. The increase of nsPEF voltage resulted in the greater SSD amplitude and shorter peak latency with the pulse of high enough voltage inducing the AP with nearly zero delay (deep blue trace). The trace of 1.47 kV/cm stimulation (light blue), which did not evoke an AP, demonstrated only SSD with the time-course corresponding to that of SSD preceding and following the APs in the other traces. Additional transient afterdepolarization waves (black arrows) were found in suprathreshold stimulations. C) An example of SSD time-course on a longer time scale, within 50 s after a 200 ns, 1.8 kV/cm pulse. AP is manifested as an abrupt fluorescence step at 10 s followed by an SSD wave lasting tens of seconds.

Figure 2.

Traces of the response to 10 consecutive nsPEF stimulations at the excitation threshold level (1.74 kV/cm, 200-ns pulse duration) in a representative cell. Note fluctuating peak latency, SSD preceding APs, and frequent transient afterdepolarizations. The red dotted line indicates the time of pulse delivery.

Figure 3.

Effect of inhibitors and external Ca²⁺ on membrane potential response to nsPEF stimulation. Individual traces are in grey and the averaged curves are in black. Tetrodotoxin (TTX) consistently blocked AP generation, though slow active responses (possibly due to calcium channel activation) could still be present. Inhibition of L-type calcium channels by verapamil, inhibition of Na⁺/Ca²⁺ exchanger by SEA0400, as well as nsPEF stimulation in Ca²⁺-free Tyrode solution all attenuated the SSD but did not fully block it (see also Fig. 4).

Figure 4.

A) SSD amplitude in cells stimulated by 1.26-1.68 kV/cm 200 ns duration pulses in presence of drugs modifying sodium and calcium currents. The amplitudes of SSD were measured as an increase of fluorescence after pulse application (the mean for the 50-130 ms afterpulse period) in respect to the fluorescence prior to pulse application (the mean for the 10 ms prepulse period). For the purpose of the calculations, parts of recordings containing APs in the abovementioned period were manually cut out of traces. B) AP threshold electric field strength in the normal Tyrode solution and under changes of cellular calcium transport

by the presence of the same drugs as in panel A (except TTX shown to inhibit AP generation). P values were calculated by one-way Dunnett's test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

nsPEF evokes Ca^{2+} transients in Tyrode solution (A), in the presence Na^{+} and Ca^{2+} channel blockers (B), but not in the absence of extracellular Ca^{2+} (C). Shown are traces of Fluo-4 fluorescence in individual cells (gray) and their average (black); some transients are truncated. A single to 200-ns stimulus of the indicated intensity was delivered at 50 s into the recording (vertical dashed line). Cell membrane electroporation is a likely cause of Ca^{2+} transients observed in the presence of channel inhibitors (B). The lack of responses in a

Ca²⁺-free solution (C), despite applying higher strength stimuli, rules out possible role of SR electroporation as a cause of Ca²⁺ response.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1.

Incidence of transient afterdepolarization waves under different conditions with nsPEF and conventional stimulation at the threshold electric field strength.

Condition (pulse duration)	Incidence, %	Total cell number	P value vs normal Tyrode (200 ns)
Normal Tyrode (200 ns)	66	106	
Verapamil (200 ns)	22	23	<0.001
Ca-free solution (200 ns)	21	28	<0.001
SEA0400 (200 ns)	54	28	0.305
Normal Tyrode (200 μ s)	8	12	0.001