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Diffuse, non-polar electropermeabilization and reduced propidium uptake distinguish the effect of nanosecond electric pulses

Iurii Semenov, Christian Zemlin, Olga N. Pakhomova, Shu Xiao, Andrei G. Pakhomov

Abstract

Ca^{2+} activation and membrane electroporation by 10-ns and 4-ms electric pulses (nsEP and msEP) were compared in rat embryonic cardiomyocytes. The lowest electric field which triggered Ca^{2+} transients was expectedly higher for msEP (36 kV/cm) than for nsEP (0.09 kV/cm) but the respective doses were similar (190 and 460 mJ/g). At higher intensities, both stimuli triggered prolonged firing in quiescent cells. An increase of basal Ca^{2+} level by >10 mM in cells with blocked voltage-gated Ca^{2+} channels and depleted Ca^{2+} depot occurred at 63 kV/cm (nsEP) or 0.14 kV/cm (msEP) and was regarded as electroporation threshold. These electric field values were at 150–230% of stimulation thresholds for both msEP and nsEP, notwithstanding a 400,000-fold difference in pulse duration. For comparable levels of electroporative Ca^{2+} uptake, msEP caused at least 10-fold greater uptake of propidium than nsEP, suggesting increased yield of larger pores. Electroporation by msEP started Ca^{2+} entry abruptly and locally at the electrode-facing poles of cell, followed by a slow diffusion to the center. In a stark contrast, nsEP evoked a “supra-electroporation” pattern of slower but spatially uniform Ca^{2+} entry. Thus msEP and nsEP had comparable dose efficiency, but differed profoundly in the size and localization of electropores.

1. Introduction

During the last decade, the research into bioeffects of intense, nanosecond duration electric pulses (nsEP) has been growing exponentially. The principal directions of this research are the lethal cell damage by nsEP and its mechanisms [1–6], with emerging applications in cancer and tissue ablation [7–11], and the biophysical mechanisms of membrane permeabilization by nsEP, properties of electropores, and their impact on cell function [4,12–16]. Recently, more studies have focused on cell stimulation and Ca^{2+} activation by nsEP, potentially leading to some unique physiological and medical applications, including heart pac- 

Abbreviations: CPA, cyclopiazonic acid; msEP, millisecond electric pulses; nsEP, nanosecond electric pulses; Pr, propidium; TMP, transmembrane potential.

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suggested that nsEP can excite nerve and muscle cells just like conventional micro- and millisecond electric shocks [17,34,35], but the mechanism underlying the excitation by nsEP was not explained or explored. Other studies [17] suggested that excitation may in part be mediated by electroporation (which leads to the loss of the resting potential, depolarization, and channel opening).

Since “short” nsEP increase TMP without movement and redistribution of mobile charges, their effect should be less restricted to electrode-facing poles of the cell. Pore enlargement presumably occurs during the imposed pulse [36], and shorter pulses can reasonably be expected to produce smaller pores. Indeed, modeling of nsEP interaction with cells predicted diffuse electroporation pattern throughout the plasma membrane and intracellular membranes, termed “supra-electroporation”, and the calculated diameter of pores was smaller than in the conventional electroporation [30,37,38]. Experimental studies confirmed poration of intracellular organelles by nsEP [9,13,20,39,40] and smaller pore size [14,36,41]. However, the diffuse, non-polar pattern of pore formation has not been demonstrated by direct experiments, which made it one of the goals of the present work.

We focused on studying the excitation and electroporation in isolated cardiomyocytes, considering both the shortage of data about nsEP effects in excitable cells, and the potential benefit of nsEP technology for cardiac defibrillation. Since at least some adverse effects of defibrillation shocks are attributed to electroporation [42–46], the formation of only smaller pores by switching from msEP to nsEP may reduce damage and cell loss. The undesired transport of solutes through electropores is further reduced by essentially eliminating the electrophoretic component [37]. The excitation which relies on the dielectric polarization should lead to deeper penetration and more uniform activation of tissue [9,22,24,25,30,32], both being crucial advantages for defibrillation. While these benefits come at the expense of the reduced amplification factor, which for nsEP is from tens to hundreds [22,24,31], higher intensity of shocks will not necessarily translate into higher energy, as the energy losses to move free charges towards the cell membrane are eliminated.

To our knowledge, nsEP effects on cardiomyocytes were analyzed in just a single peer-reviewed study, which reported both Ca2+ transients and Ca2+ waves induced by 4-ns stimuli [17]. The authors concluded that at least for certain nsEP parameters, these Ca2+ responses are probably mediated by nanoporation of sarcolemma. They employed multi-pulse stimulation protocols which complicated the interpretation of results (repetitive nsEP at 2 Hz; or three 1-ms, 2.4 kV/cm stimuli followed by either a single nsEP or a 10-kHz nsEP train), especially considering that 2.4 kV/cm is well above the electroporation threshold for 1-ms pulses [47–49] and that intense instant heating was likely (up to 20 °C per one msEP, based on the dose and adiabatic heat calculation [50,51]). In this work, we compared the stimulation and electroporation effects of individual 10 ns and 4 ms stimuli for different pulse intensities.

2. Materials and methods

2.1. Cell culture

Embryonic rat cardiac myocytes, the culture medium, and its supplements were purchased from Lonza (Walkersville, MD) and handled according to supplier recommendations. Cells were seeded on “0” thickness glass coverslips coated with nitrocellulose and incubated at 37 °C with 5% CO2 in air, in RGM medium supplemented with 7.5% horse serum, 7.5% fetal bovine serum, 0.1% of pre-mixed gentamicin/amphotericin-B solution, and 200 μM of 5-bromo-2’-deoxyuridine. To prepare coverslip coating solution, a nitrocellulose filter paper (Invitrogen, Eugene, OR) was dissolved in methanol (0.1 cm2/10 ml). Every 3 days, 50% of the growth medium was replaced by fresh one. Cells on coverslips did not propagate and were used up to three weeks after thawing.

2.2. Reagents and solutions

Fura-2 pentapotassium salt, Fura-2/AM, and Pluronic F-127 (20% solution in DMSO) were purchased from Life Technologies (Grand Island, NY). Verapamil and cyclosporin acid (CPA) were obtained from Tocris Bioscience (Minneapolis, MN). Other chemicals were from Sigma-Aldrich (St. Louis, MO). During experiments, cells were continually perfused at 0.5 ml/min (or 10 ml/min for faster drug delivery) with a physiological solution containing (in mM): 140 NaCl, 5.4 KCl, 1.5 MgCl2, 2 CaCl2, 10 glucose, and 10 HEPES (pH 7.3, 300–310 mOsm/kg). For Ca2+-free conditions, CaCl2 was replaced with 2 mM Na-EGTA.

2.3. Measurement of cytosolic Ca2+ concentration

The detailed procedures employed for loading cells with Fura-2 and dye calibration were reported earlier [13,20]. In brief, cells were loaded with the dye for 30 min at room temperature, in the dark, in the physiological solution supplemented with 5 μM Fura-2/AM and 0.02% of Pluronic F-127. After loading, the coverslip was placed in a glass-bottomed perfusion chamber mounted on an IX71 microscope (Olympus America, Center Valley, PA) and washed with the physiological solution for 15 min to allow for deesterification of the dye. Fura-2 fluorescence was measured using an ET FURA2 filter set (Chroma Technology, Bellows Falls, VT) and a UAp0N340 40 ×/1.35 objective (Olympus). A fast wavelength switcher Lambda DG4 (Sutter Instruments, Novato, CA) was employed to excite the dye alternatively at 340 and 380 nm and fluorescence was recorded at 510 nm. Images were acquired in a streaming mode (20 ms exposure, 25 image pairs/s) using Metafluor v.7.5 software (Molecular Devices, Sunnyvale, CA) and iXon Ultra 897 EM CCD camera (Andor Technology, Belfast, UK).

2.4. Measurement of propidium (Pr) uptake

The perfusion chamber was filled with the physiological solution containing 3 μg/ml of Pr iodide and perfusion was turned off. Pr becomes highly fluorescent upon entering the cell and binding to nucleic acids, but cannot enter the cell through the intact plasma membrane. Therefore, the gain in Pr emission is commonly used to quantify the disruption of the plasma membrane barrier function by electroporation [14,27,52–55]. Emission of Pr was recorded using a TRITC filter cube (Olympus) and the iXon Ultra 897 camera (100 ms exposure, 0.9 images/s). The recording began 1 min prior to electroporation and continued for 9 min after it; the gain in fluorescence by the end of recording was used as a measure of Pr uptake.

2.5. Exposure to nsEP and msEP

The technique of electric field delivery to individual cells was the same as reported previously [4,13–15,20,55]. Trapezoidal pulses of up to 20 kV amplitude and approximately 10 ns duration (at 50% height) were produced by a model FPG 20-1NM pulse generator (FID GmbH, Burbach, Germany). Rectangular 4-ms pulses were produced by a Grass S88 Stimulator (Grass Instrument, Quincy, MA). Typical pulse shapes of nsEP and msEP are presented in the inset of Fig. 1.

Pulses were delivered to selected cells on a coverslip with a pair of tungsten rod electrodes (0.1 mm diameter). The pulse shapes and amplitudes were monitored with a TDS 3052 oscilloscope (Tektronix, Beaverton, OR). Electrodes were positioned precisely at 50 μm above the coverslip surface using an MPC-200 robotic manipulator (Sutter, Novato, CA). To deliver nsEP at the lowest tested electric field of 25 kV/cm, the electrodes were raised to 70 μm. Cells selected for exposure were in the middle of the 0.24-mm gap between the tips of the electrodes. Pulses were triggered externally and synchronized with image acquisition and bath buffer exchanges by a TTL pulse protocol using Digidata 1440A board and Clampex v. 10.2 software (Molecular Devices, Sunnyvale, CA). The electric field at the location of the cells
of cytosolic Ca$^{2+}$ to the basal level could take minutes, followed by the restoration of quiescent state and generation of “regular” all-or-none Ca$^{2+}$ transients in response to stimuli of the near-threshold intensity (Fig. 2A). In other cells, spontaneous Ca$^{2+}$ oscillations persisted even after the restoration of the basal Ca$^{2+}$ level; moreover, the oscillations recovered after incubation with 10 μM nifedipine or extracellular Ca$^{2+}$ removal (Fig. 2B,C). Thus, the lasting Ca$^{2+}$ oscillations relied on the spontaneous opening of L-type Ca$^{2+}$ channels, and the “active” condition of the cell was preserved regardless of whether it was actually firing or not. A detailed analysis of mechanisms involved would require a separate study; here we would only like to note the qualitative similarity of nsEP and msEP effects as the intensity of the stimuli was increased beyond the near-threshold levels.

3.3. Dose efficiency of nsEP and msEP for electroporation of the sarcolemma

In order to quantitate the electroporative Ca$^{2+}$ entry, we used a cocktail of 10 μM verapamil (to block L-type Ca$^{2+}$ channels of the sarcolemma), 10 mM caffeine (to deplete Ca$^{2+}$ from the sarcoplasmic reticulum by stimulating ryanodine receptors), and 10 μM of CPA (to block re-uptake of Ca$^{2+}$ to the reticulum). This cocktail triggered a brief Ca$^{2+}$ elevation followed by a drop and stabilization below the basal level (Fig. 3). Concurrently, the cocktail fully blocked Ca$^{2+}$ transients in response to nsEP or msEP of the near-threshold electric field. However, an increase of the stimulus strength evoked Ca$^{2+}$ responses of a different type: (1) their amplitude increased gradually as the stimulus strength was increased, (2) Ca$^{2+}$ concentration could continue to increase for tens of seconds with or without recovery, and (3) when present, Ca$^{2+}$ recovery to the basal level was slow (Figs. 3 and 4A). This response was abolished in a Ca$^{2+}$-free medium (data not shown) and was concluded to be a result of electroporation of the plasma membrane.

The smallest Ca$^{2+}$ increase that could be reliably resolved in our experiments was about 10 nM (a horizontal dotted line in Fig. 4). Thus the electroporation thresholds were 0.14 and 63 kV/cm for msEP and nsEP, respectively (Fig. 4A). Despite this 500-fold difference, the threshold electroporation dose was 2-fold smaller for nsEP (0.5 J/g) than for msEP (1.2 J/g).

Above the threshold, the amplitude of Ca$^{2+}$ rise increased as a power function of the stimulus intensity. In order to compare the stimulation and electroporation thresholds, we (a) normalized the electroporating voltages to the near-threshold stimulation electric field values (0.09 kV/cm for msEP and 36 kV/cm for nsEP), or (b) normalized the electroporating voltages to the highest known
subthreshold electric field (0.05 kV/cm for msEP and 25 kV/cm for nsEP). These steps have yielded, respectively, the lower and the higher boundaries of electroporation thresholds estimated relative to the stimulation thresholds.

When the msEP and nsEP intensities were normalized as in step (a) above, the Ca\(^{2+}\) uptake values overlapped and could be approximated with a common power fit, with a high coefficient of determination (\(R^2 = 0.95\), Fig. 4B). The intercept of this best fit line with the 10-nM detection limit at 150% (Fig. 4B) can be regarded as a lower boundary of the electroporation threshold for both msEP and nsEP. For normalization as in step (b), the normalized Ca\(^{2+}\) uptake values also overlapped and could be approximated with a common power fit (\(R^2 = 0.91\); data not shown). From this fit, the upper boundary of the electroporation threshold was estimated at 230%.

Thus, for both msEP and nsEP, electroporation occurred at intensities of only 150–230% of the stimulation threshold (or at doses between 200% and 500%), which was a striking similarity for stimuli differing in duration by almost 6 orders of magnitude.

### 3.4. Different size of pores opened by nsEP and msEP

If the safety factor of electrostimulation is defined simply as a ratio of electroporating and stimulating voltages (or doses), our data would suggest no impact from changing the pulse duration. At the same time, previous studies as well as in silico models found that shorter electric pulses produce fewer large-diameter pores [12,14,30,36] and should be less damaging. Measuring the electroporative entry of a small cation such as Ca\(^{2+}\) does not necessarily reveal the difference in pore size, hence it was probed with a larger Pr cation [15,54].

From plots in Fig. 4A, we identified the electric field intensities of msEP and nsEP which caused practically the same Ca\(^{2+}\) response: 0.14 and 63 kV/cm; 0.55 and 135 kV/cm; and 1.1 and 270 kV/cm. In separate experiments, we measured Pr uptake and plotted it against the Ca\(^{2+}\) uptake at the respective stimulus strength. Fig. 5 shows that for nsEP and msEP treatments which caused the same Ca\(^{2+}\) rise, the msEP treatment caused at least a 10-fold greater Pr uptake. This result indicates that msEP indeed opened more of the larger, Pr-permeable pores. Perhaps the fraction of Pr-permeable pores was small when compared to the entire pore population, and therefore had little impact on Ca\(^{2+}\) uptake which entered the cell through a much larger population of pores. This conclusion is similar to the one made earlier when comparing Pr and water uptake caused by 60- and 600-ns electric pulses [14]. Notwithstanding the relatively small number of larger-size pores, the physiological consequences of their formation can be significant. For example, the presence of even a small population of larger-size pores was implicated as a major reason why cell survival in cells exposed...
to high-intensity 300-ns, 2-μs, or 9-μs electric pulses was much lower than after 10-ns pulse treatments at the same dose [1]. Thus, despite the similarity of electroporation thresholds with respect to the stimulation thresholds, nsEP will likely cause less damage to cells by not opening larger electropores in the plasma membrane.

### 3.5. Supra-electroporation of sarcolemma by nsEP

As discussed above, with long electric pulses and capacitive charging of the cell membrane, the critical TMP builds up primarily at cell poles facing the electrodes. With nsEP being too brief to move ions to charge

![Fig. 3](image-url)  
**A** cocktail of caffeine, CPA, and verapamil fully blocks Ca$^{2+}$ transients in response to previously effective stimuli and enables the observation of electroporative Ca$^{2+}$ entry after more intense stimuli. A and B: representative experiments with msEP and nsEP, respectively. The perfusion with the cocktail (horizontal bar) transiently increases the cytosolic Ca$^{2+}$, followed by its drop below the basal level. An inset zoom (B) shows a small Ca$^{2+}$ response with 10× magnification. See Fig. 2 for other details.

![Fig. 4](image-url)  
**B** The amplitude of electroporative Ca$^{2+}$ entry as a function of stimulus intensity for msEP and nsEP. A: 10-ns pulses require much higher electric field to cause electroporation than 4-μs pulses. Measurements were performed in cells blocked with 10 mM caffeine, 10 μM CPA, and 10 mM verapamil. The horizontal dotted line at 0.01 μM delimits the lowest detectable Ca$^{2+}$ increase, so the datapoints falling below this line are considered subthreshold for electroporation. Mean values ± s.e. for 5–8 experiments; the error bars may not be visible when they are smaller than the central symbol. Ca$^{2+}$ entry for datapoints above the dotted line was statistically significant at p \( < 0.05 \) or better. The labels next to the datapoints are the respective dose values, in J/g. The dashed lines are the best power function fits of data above 0.01 μM. Two open symbols at 0 Ca$^{2+}$ uptake are the values subthreshold for stimulation (in cells not blocked with the drugs). B: Same data expressed in % to the minimum electric field known to be effective for stimulation of unblocked cells, namely 0.09 and 36 kV/cm for msEP and nsEP, respectively. The dashed area is the common power function fit and the shaded corridor sets the limits of a 95% confidence interval. See text for more details.
the membrane, and relying on the dielectric polarization instead, the electroporation by nsEP should be less restricted to cell poles. Simulation models predicted a widespread, diffuse pattern of supra-electroporation [30,38], which however has not been demonstrated by direct measurements.

To compare the localization of electroporation by msEP and nsEP, we chose larger cells (50–100 μm in diameter) which would allow for better spatial resolution of Ca²⁺ gradients by fluorescent imaging. The non-electroporative mechanisms of Ca²⁺ response to electric stimuli were blocked with the drug cocktail described above. Electroporated areas of the cell membrane were recognized by the route of Ca²⁺ entry as monitored by a time-lapse imaging. While we did not block non-selective cation channels, they are not voltage-gated and were not expected to be activated directly by msEP or nsEP.

The experiments revealed a striking difference in the spatial patterns of nsEP and msEP effects (Figs. 6 and 7). With 4-ms pulses, Ca²⁺ entered from the poles (more from the anodic pole) and diffused to the center of the cell for seconds after the pulse. In Fig. 7, this pattern of entry is manifested by the reduced and delayed Ca²⁺ increase in the center of the cell compared to the electrode-facing poles. The early Ca²⁺ entry at the anodic pole could be assisted by electrophoresis during the pulse, which explains a brief dip in the course of Ca²⁺ increase. In contrast to msEP, 10-ns pulses caused a gradual, slow increase of Ca²⁺ in the entire volume of the cell, without any specific localization and
synchronously in all measured regions.

In near-threshold intensities, both nsEP and msEP triggered Ca\(^{2+}\) transients in rat embryonic cardiomyocytes, apparently by activating voltage-gated channels without electroporation. The electroporation threshold for both stimuli was at just 150–230% of the stimulation threshold (200–500% when comparing the dose). Such similarity was unexpected from stimuli which differ by almost six orders of magnitude.

4. Conclusions

At near-threshold intensities, both nsEP and msEP triggered Ca\(^{2+}\) transients in rat embryonic cardiomyocytes, apparently by activating voltage-gated channels without electroporation. The electroporation threshold for both stimuli was at just 150–230% of the stimulation threshold (200–500% when comparing the dose). Such similarity was unexpected from stimuli which differ by almost six orders of magnitude. At the same time, we experimentally established two distinguishing features of electroporation by nsEP, namely the reduced formation of larger-size pores and non-polar, diffuse distribution of electropores over the cell body. These distinguishing features may be beneficial for medical applications such as defibrillation (by reducing the harmful side effects from electroporation and by enabling more uniform tissue penetration) and warrant more focused studies at tissue and organ levels.

### References


