Biochimica et Biophysica Acta 1848 (2015) 2118–2125

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

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ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form 31 May 2015
Accepted 15 June 2015
Available online 22 June 2015

Keywords:
Electroporation
Electropermeabilization
Calcium activation
Nanosecond electric pulses
Cardiomyocytes

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 nsEP (36 kV/cm) than for msEP (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 nM 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 nsEP and msEP 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 pacing, defibrillation, and stimulation of neurosecretion and other functions [13,17–21]. However, the mechanisms of cell excitation and Ca^{2+} mobilization by nsEP remain uncertain.

With conventional electrostimulation using "long" (micro- and millisecond) pulses, the externally applied electric field moves ions in extra- and intracellular electrolytes, whereas the cell membrane acts as a barrier. The resulting build-up of the electric potential across the membrane amplifies the externally applied electric field (the process called Maxwell–Wagner ionic polarization), with the amplification factor for mammalian cells being on the order of thousands [22–24]. This amplification enables the excitation and electroporation by external electric fields many orders of magnitude weaker than the natural electric field across the cell membrane. External fields impose the highest transmembrane potential (TMP) at cell poles facing stimulating electrodes, and membrane depolarization at the cathode-facing pole leads to the opening of voltage-gated channels and excitation. In contrast, electroporative TMP levels can be reached faster at the anode-facing pole where the resting membrane potential adds to the external electric field. The polar pattern of membrane permeabilization by individual millisecond pulses (msEP) and shorter pulses (down to at least 600 ns) has been routinely detected by the polar entry of marker dyes and ions (propidium, ethidium, YO-PRO-1, and Ca^{2+}) [25–28]. The entry could be higher from either anodic or cathodic pole, depending on the marker and other factors, including the different pore size at the two poles [27]. Polar dye entry was also reported after high-rate trains of multiple pulses as short as 4 ns [29].

Stimuli shorter than 100–200 ns may be too brief to cause ionic polarization, but can induce TMP needed for electroporation by a dielectric polarization mechanism [22,24,25,30–32]. However, the amplification factor is smaller and the stimulus strength needs to be increased accordingly. Moreover, such stimuli appear too brief to shift the voltage sensor of voltage-gated channels across the plasma membrane and cause a series of conformation changes leading to the channel opening (which takes 10–100 μs [33]). Still, isolated reports

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

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http://dx.doi.org/10.1016/j.bbamem.2015.06.018
0005-2736/

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 Ca^{2+} transients and Ca^{2+} waves induced by 4-ns stimuli [17]. The authors concluded that at least for certain nsEP parameters, these Ca^{2+} 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 conditions and in a wide range of 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% CO_2 in air, in RCGM 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 cm^2/10 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 cyclopiazonic 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 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.3, 300–310 mOsm/kg). For Ca^{2+}-free conditions, $CaCl_2$ was replaced with 2 mM Na-EGTA.

2.3. Measurement of cytosolic Ca^{2+} 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 UApoN340 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