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Electroporation by subnanosecond pulses

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ABSTRACT

Electropermeabilization of cell membranes by micro- and nanosecond-duration stimuli has been studied extensively, whereas effects of picosecond electric pulses (psEP) remain essentially unexplored. We utilized whole-cell patch clamp and Di-8-ANEPPS voltage-sensitive dye measurements to characterize plasma membrane effects of 500 ps stimuli in rat hippocampal neurons (RHN), NG108, and CHO cells. Even a single 500-ps pulse at 190 kV/cm increased membrane conductance and depolarized cells. These effects were augmented by applying brief psEP bursts (5–125 pulses), whereas the rate of pulse delivery (8 Hz–1 kHz) played little role. psEP-treated cells displayed large inward current at negative membrane potentials but modest or no conductance changes at positive potentials. A 1-kHz burst of 25 pulses increased the whole-cell conductance in the range (−100)–(−60) mV to 22–26 nS in RHN and NG108 cells (from 3 and 0.7 nS, respectively), but only to 5 nS in CHO (from 0.3 nS). The conductance increase was reversible within about 2 min. Such pattern of cell permeabilization, with characteristic inward rectification and slow recovery, was similar to earlier reported effects of 60- and 600-ns pulses, pointing to the similarity of structural membrane rearrangements in spite of a different membrane charging mechanism.

1. Introduction

Application of high-voltage electric pulses (EP) of micro- or millisecond duration to living cells is a well-established technique to increase cell membrane permeability and introduce normally impermeable substances into cells [1–3]. This process, termed electroporation or electropermeabilization, has numerous applications in experimental biology, medicine, and biotechnology.

In parallel with the advancement of pulsed power engineering during the last decades, electroporation research expanded to shorter and higher amplitude electric pulses. Early work with nanosecond EP (nsEP) focused on electroporation of intracellular membranes, whereas the plasma membrane was thought to stay unaffected [4–7]. Later theoretical and experimental studies established that nsEP cause the formation of long-lived nanopores in the plasma membrane [8–11]. These nanopores had complex conductive properties, including voltage and current sensitivity, inward rectification, and ion selectivity. Interestingly, similar features could occasionally be established in cells permeabilized by ms-range EP or even subjected to hyperpolarization in a low K^+ solution [12–14]. Nanopores could supposedly be created by "long" EP either as a fraction of a mixed-size pore population, or by shrinking of larger electropores. Gd^{3+} and La^{3+} ions were effective at inhibiting or preventing pore conductance regardless of the exact permeabilization method [10,12,14–16]. Although the data suggest similar properties of nanopores produced by different treatments, this conjecture has yet to be tested by direct experiments.

Furthermore, our recent data on Ca^{2+} mobilization by picosecond-range EP (psEP) [17] could not be easily explained by existing paradigms of electropore formation. We established that Ca^{2+} activation by 500-ps, 190 kV/cm EP is mediated by opening of voltage-gated calcium channels (VGCC) and therefore does not occur in CHO cells (which do not express any VGCC). This observation contrasted numerous findings that 60-ns and longer EP do not rely on VGCC and efficiently activate Ca^{2+} in CHO cells [18–21]. Therefore, findings with psEP could be indicative of a non-conventional membrane electroporation, with pores so short-lived that usual methods of pore detection fail. One may also speculate that the lack of VGCC expression or some unknown differences in membrane composition made CHO cells less vulnerable to psEP, although it was not the case for nsEP (CHO cells showed similar sensitivity to 60- and 600-ns pulses as VGCC-expressing NG108 and GH3 cells [22,23]). Finally, one may think of VGCC activation by psEP in the absence of electroporation, just by psEP-induced

Abbreviations: EP, electric pulses; MP, membrane potential; nsEP, nanosecond electric pulses; psEP, picosecond electric pulses; VGCC, voltage-gated calcium channels; RHN, rat hippocampal neurons

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depolarization of the plasma membrane, similarly to conventional electrostimulation. However, 500-ps pulses are too short to change the membrane potential by Maxwell-Wagner polarization and have to rely on the dielectric stacking effect instead. As a result, the relaxation of the psEP-induced membrane potential is essentially instant and happens roughly 1000 times faster than movement of the voltage sensor of a VGCC; hence, direct VGCC activation by psEP not mediated by electroporation is also difficult to explain [17].

The present work was aimed at further exploring psEP-induced electroporation by electrophysiological and optical membrane potential detection techniques. Bioeffects of psEP remain essentially an uncharted territory, with current knowledge limited to a few isolated reports [17,24–26]. Below we demonstrate that even a single psEP at 190 kV/cm can permeabilize cell membrane, and that psEP- and nsEP-porated membranes share similar features. At the same time, CHO cells proved to be less sensitive to psEP than other tested cells, which contrasts earlier findings using nsEP [22,23] but is consistent with the lack of Ca^{2+} activation by psEP [17].

2. Materials and methods

2.1. Cells and media

Chinese hamster ovary cells CHO-K1 and a murine neuroblastoma-rat glioma hybrid NG108 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were propagated at 37 °C with 5% CO_2 in air according to the supplier's recommendations. CHO cells were grown in Ham's F12K medium (Mediatech Cellgro, Herdon, VA) supplemented with 10% fetal bovine serum (FBS), 100 I. U./ml penicillin and 0.1 μg/ml streptomycin. NG108 cells were cultured in Dulbecco's Modified Eagle's medium (Caisson Labs, North Logan, UT) without sodium pyruvate, supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 10% FBS, 0.2 mM hypoxanthine, 400 nM aminopterin, and 0.016 mM thymidine (without antibiotics). The media supplements were from Sigma-Aldrich (St. Louis, MO) except for the serum (Atlanta Biologicals, Norcross, GA). For the passage immediately preceding experiments, cells were transferred onto glass coverslips. Cells were used in experiments after 12–24 h of growing on the coverslips.

Dissociated E18 rat hippocampal neurons (RHN) were purchased from BrainBits LLC (Springfield, IL) and seeded on poly-D-lysine/laminin coated glass coverslips (Corning, Corning, NY) in Gibco Neurobasal medium supplemented with 50 × B-27 (20 ml/l) and 100 × Glutamax (2.4 ml/l) (all from Thermo Fisher Scientific, Waltham, MA). One half of the medium was replaced every 3 days. Neurons were used between 1 and 3 weeks in culture.

2.2. Electrophysiology

Whole-cell mode of conventional voltage clamp or current clamp (at I=0) were used to quantify psEP-induced changes in the membrane conductance and resting membrane potential (MP), respectively. The measurements were performed using Axopatch 200B amplifier, Digidata 1440 A board, and Clampex v. 10.2 software (Molecular Devices, Sunnyvale, CA). Coverslips with cells were placed in a glass-bottomed perfusion chamber (Warner Instruments, Hamden, CT) mounted on a stage of an IX71 microscope (Olympus America, Center Valley, PA). The extracellular solution contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.2). Recording pipettes were manufactured by pulling borosilicate glass (BF-150–866–10, Sutter Instrument, Novato, CA) to a tip resistance of 1–3 MOhm using a Flaming/Brown P-97 Micropipette puller (Sutter, Novato, CA), and filled with (mM): 10 NaCl, 130 KCl, 2 $CaCl_2$, 3 $MgCl_2$, 10 HEPES, and 5 K-EGTA (pH of 7.2). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

The membrane conductance measurement protocols and procedures were similar to described previously [8,9,15,27]. The positioning of the recording pipette with respect to psEP-delivering electrodes was the same as illustrated in [23], with the exception of the fact that the gap between the psEP-delivering electrodes in the current study was somewhat different. Within 1–2 min after the whole-cell configuration was established, membrane currents were measured by applying a voltage-step protocol (80-ms steps from −100 to 40 mV in 10-mV increments) from the holding potential of −80 mV. This protocol took about 3 s, and it was applied at 5 s before psEP exposure and again at 1, 5, 30, and 120 s after it. The current at each step was measured after the steady-state level was reached, i. e., at 30–50 ms into the step. Thus, we generated a series of current-voltage (I-V) curves to compare the whole-cell currents before psEP exposure and at indicated time intervals after it. The whole-cell conductance was measured by linear fitting of the I-V curves in individual cells in the range from −100 to −60 mV.

The membrane voltages reported in this paper have not been corrected for the junction potential (which equaled 4.2 mV).

2.3. Optical membrane potential monitoring with Di-8-ANEPPS

To load Di-8-ANEPPS into cell plasma membrane, coverslips with cells were incubated in the extracellular solution with 20 μM of the dye for 45 min at 4 °C. The coverslips were transferred into the glass-bottomed perfusion chamber and the excess dye was rinsed off.

The MP was measured by ratiometric imaging, which enabled a more reliable calibration, better signal-to-noise ratio, and reduced the impact of bleaching. The dye was excited alternately in 5-ms windows at 440 and 530 nm using fast wavelength switcher Lambda DG4 (Sutter Instruments, Novato, CA). We utilized a U-N71006 Di-8-ANEPPS filter set (Chroma Technology, Bellows Falls, VT) and a PlanApo N 60 × /1.42 objective (Olympus). Emission was measured at 605 nm with an iXon Ultra 897 back-illuminated CCD Camera (Andor Technology, Belfast, UK). Dye emission ratio was calibrated against the membrane potential in voltage-clamped NG108 cells.

MP measurements with the dye typically began 2 s prior to psEP delivery and continued for 10 s after the exposure, at 100 image pairs/s. The image acquisition and on-line data analysis were accomplished with Metafluor v.7.5. (Molecular Devices). An FFT filter utility of Origin 8.0 (OriginLab Corporation, Northampton, MA) was employed for offline noise reduction.

2.4. psEP exposure and dosimetry

We utilized the same psEP delivery and measurement techniques as reported recently [17]. In brief, pulses were produced by an FPG 20–1 PM generator (FID GmbH, Burbach, Germany). They were triggered externally and synchronized with image acquisitions or voltage step protocols using Digidata 1440 A board. The exact timing of psEP delivery, pulse rate, and the number of pulses were all programmed in Clampex software.

The pulses were sent to a 4 GHz, 20 Gs/s TDS7404 oscilloscope (Tektronix, Beaverton, OR) and to a π-network intended to absorb reflections from the load. psEP were delivered to cells in the bath by means of a pair of tungsten rods (100 μm diameter, 170 μm gap) at the end of a 50-Ω RG316 coaxial cable. The electrode assembly was driven by an MPC-200 robotic manipulator (Sutter Instruments, Novato, CA), to place the tips of the rods precisely at 50 μm