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Expression of voltage-gated calcium channels augments cell susceptibility to membrane disruption by nanosecond pulsed electric field

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ABSTRACT

We compared membrane permeabilization by nanosecond pulsed electric field (nsPEF) in HEK293 cells with and without assembled CaV1.3 L-type voltage-gated calcium channel (VGCC). Individual cells were subjected to one 300-ns pulse at 0 (sham exposure); 1.4; 1.8; or 2.3 kV/cm, and membrane permeabilization was evaluated by measuring whole-cell currents and by optical monitoring of cytosolic Ca^{2+}. nsPEF had either no effect (0 and 1.4 kV/cm), or caused a lasting (> 80 s) increase in the membrane conductance in about 50% of cells (1.8 kV/cm), or in all cells (2.3 kV/cm). The conductance pathway opened by nsPEF showed strong inward rectification, with maximum conductance increase for the inward current at the most negative membrane potentials. Although these potentials were below the depolarization threshold for VGCC activation, the increase in conductance in cells which expressed VGCC (VGCC+ cells) was about twofold greater than in cells which did not (VGCC− cells). Among VGCC+ cells, the nsPEF-induced increase in membrane conductance showed a positive correlation with the amplitude of VGCC current measured in the same cells prior to nsPEF exposure. These findings demonstrate that the expression of VGCC makes cells more susceptible to membrane permeabilization by nsPEF. Time-lapse imaging of nsPEF-induced Ca^{2+} transients confirmed permeabilization by a single 300-ns pulse at 1.8 or 2.3 kV/cm, but not at 1.4 kV/cm, and the transients were expectedly larger in VGCC+ cells. However, it remains to be established whether larger transients reflected additional Ca^{2+} entry through VGCC, or were a result of more severe electropermeabilization of VGCC+ cells.

1. Introduction

Exposure of cells to nsPEF causes formation of permeable structures in the cell membrane, often referred to as “nanopores” [1–7]. Because of their long lifespan (minutes), nanopores affect virtually all aspects of cell physiology. One of the first, and arguably the most significant events that occur after nsPEF-induced nanopore formation is an increase in the cytosolic free Ca^{2+} [8–16]. Ca^{2+} is a universal second messenger which controls a variety of processes in the cell, and its increase can activate multiple downstream signaling cascades [17]. The ability of nsPEF to elevate cytosolic Ca^{2+} by nanopore opening could become a promising approach for non-chemical triggering of Ca^{2+}-signaling in various cell types. Ca^{2+} activation by nsPEF can potentially find applications for heart pacing, defibrillation, and stimulation of neurosecretion and other functions [8,13,14,18–21]. Therefore, detailed understanding of the mechanisms by which nsPEF affects the intracellular Ca^{2+} dynamics is of fundamental importance for biomedical science and medicine.

Voltage-gated Ca^{2+} channels (VGCC) are one of the major transmembrane pathways for Ca^{2+} to enter the cell [22] and one of the most likely targets for nsPEF stimulation. These channels are activated by membrane depolarization, since they are equipped with a voltage sensor which detects changes in the membrane potential [22]. VGCC are ubiquitously expressed in excitable cells and are essential for regulation of Ca^{2+} homeostasis and excitability. In spite of their fundamental significance for cell physiology only a handful of studies explored the effects of nsPEF on VGCC [8,20,23,24]. In particular, it remains controversial whether nsPEF can activate VGCC directly, or there is always an intermediate step of membrane depolarization due to the leak currents in an electroporated membrane. In bovine chromaffin cells, 5-ns pulses at 50 kV/cm caused VGCC activation as a result of membrane depolarization by Na^+ influx [8]. In adult rat cardiomyocytes, 4-ns stimuli at 10–80 kV/cm caused Ca^{2+} mobilization by VGCC activation, which was caused by membrane depolarization both

Abbreviations: nsPEF, nanosecond pulsed electric field; VGCC, voltage-gated calcium channels; VGCC−, cells which do not express VGCC; VGCC+, cells which express VGCC

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directly (by the nsPEF-imposed electric potential) and indirectly, as a result of electroporation and the resulting loss of the resting membrane potential. At the same time, in embryonic rat cardiomyocytes the threshold for eliciting Ca^{2+} transients (which were dependent on VGCC opening) by a 10 ns pulse was 36 kV/cm [11], which was significantly lower than the electroporation threshold (63 kV/cm). Aside from the immediate VGCC activation by membrane depolarization, nsPEF-treated cells experienced a long-lasting inhibition of VGCC, and the effect was apparently independent of electroporation [23]. Other studies, which compared nanoelectroporation by sub-nanosecond PEF in several different cell types, pointed to differences in electroporation between cells which constitutively express VGCC (such as neuroblastoma, neurons, and pituitary cells) and those which do not (CHO cells) [9,24]. For example, trains of up to 100 pulses (0.5 ns, 190 kV/cm) elicited no response in CHO cells, whereas even a single pulse of the same duration and amplitude evoked Ca^{2+} transients in NG108 and GH3 cells [24]. Likewise, a 1-kHz burst of 25 pulses increased the whole-cell conductance to 22–26 nS in NG108 and in rat hippocampal neurons, but only to 5 nS in CHO cells [9]. These results were indicative of a non-conventional membrane electroporation which involves membrane proteins, rather than just lipids [9,24]. However, no conclusive connection could be made between VGCC expression and electroporation, because the difference in VGCC expression was just one of uncountable physiological differences between these different cell lines.

In order to isolate the role of VGCC, any other differences between cells which do and do not express VGCC (VGCC+ and VGCC− cells) should be reduced to the minimum. In this study, this goal was accomplished by using HEK293 cells (which are naturally VGCC−) and transiently transfecting them to express VGCC. In practice, any cell sample on the coverslip was a mixture of successfully transfected VGCC+ cells and VGCC− cells which underwent the same treatments, but did not get transfected. We utilized patch-clamp electrophysiology and fluorescent Ca^{2+} imaging to analyze electroporation and other effects of 300-ns PEF and relate them to VGCC expression. In each individual cell, the level of VGCC expression was evaluated by either patch clamp or depolarization with KCl (as described below in Materials and methods), and cells regarded as VGCC+ and VGCC− were assigned to different groups for the analysis of nsPEF effects.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained in Eagle's Minimum Essential Medium (EMEM) with 1.5 g/l sodium bicarbonate, non-essential amino acids, L-glutamine and sodium pyruvate (Mediatech Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (certified OneShot FBS, Life Technologies, Grand Island,NY), 100 IU/ml penicillin, and 0.1 μg/ml streptomycin (Mediatech Cellgro) at 37 °C, 5% CO_2.

cDNAs coding for rat CaV1.3, CaVb and a2d1 rat calcium channel subunits were obtained from Addgene (Cambridge, MA, Addgene plasmid numbers 26576, 26574, 58726 respectively). All the plasmids were provided to Addgene by Drs. D. Lipscombe (Brown University, Providence, RI) and A. Dolphin (University College London, London, UK) and are described in details elsewhere [25,26]. pDNA coding for fluorescent protein mCherry was obtained from Clonetech (Takara, Mountain View, CA, plasmid number 632524). For transfection experiments, channel's cDNAs were mixed at 1:1:1 molar ratio and mCherry added at 1/10 w/w.

Sixteen hours before transfection HEK293 cells were plated in 15-mm wells of 4-well tissue dish (Fisher Scientific, Asheville, NC) at 60–70% confluency. Next day, cell were co-transfected with multi-plasmid cDNA mix using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA), and left in a CO_2 incubator at 37 °C. The culture medium was replaced with new medium after 6 h. Next day, the cells were detached from the dish with TrypLE ™ Select (Gibco, Gaithersburg, MD) and seeded on laminin-coated coverslips (neuVitro, Vancouver, Canada). Electrophysiological recordings and fluorescent microscopy assays were performed 2–4 days after transfection. All experiments were done at room temperature (22 ± 2 °C).

2.2. Exposure to nsPEF

Exposure to nsPEF and dosimetry were described in detail recently [16]. Briefly, trapezoidal unipolar 300-ns pulses were produced by a custom-built MOSFET-based generator [27] upon delivery of a TTL trigger pulse from pClamp software via a Digidata 1322A output. Synchronization of nsPEF exposure with image acquisition and patch clamp data collection was also accomplished via pClamp protocols. Pulses were delivered to a selected cell or a small group of cells with a pair of tungsten rod electrodes (100 μm diameter, 150–250 μm gap). The electrodes were positioned 50 μm above the coverslip using a robotic manipulator (MP-225, Sutter Instruments, Novato, CA). In all experiments, we delivered a single 300-ns pulse at 0 kV/cm (sham exposure), 1.4, 1.8, or 2.3 kV/cm. The electric field at the cell location was calculated as described previously [16] by 3D numerical simulations using a finite element analysis software COMSOL Multiphysics, release 5.0 (COMSOL Inc., Stockholm, Sweden). The exact shape and amplitude of nsPEF were monitored using a TDS 3052B oscilloscope (Tektronix, Beaverton, OR).

2.3. Cell imaging and calcium transient measurements

Cytosolic Ca^{2+} was monitored by fluorescence imaging with Fluo-4 (ThermoFisher Scientific, Waltham, MA). Cells were loaded with the dye by incubation for 20 min with 5 μM of Fluo-4/AM and 0.02% of Pluronic F-127 in a physiological solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 Glucose (pH 7.4 with NaOH). The coverslips were rinsed and transferred into a glass-bottomed chamber (Warner Instruments, Hamden, CT) mounted on an Olympus IX81 inverted microscope equipped with an FV1000 confocal laser scanning system (Olympus America, Center Valley, PA). The chamber was filled with the same physiological solution, but $CaCl_2$ concentration was increased to 5 mM to enhance Fluo-4 response. All chemicals and solutions were from Sigma-Aldrich (St. Louis, MO). The osmolality of the physiological solution was between 290 and 310 mOsm/kg, as measured with a freezing point Advanced™ Micro Osmometer (Model 3300, Advanced Instruments, Inc., Norwood, MA).

Differential-interference contrast (DIC) and fluorescence images were taken with a 40×, NA 0.95 dry objective. Fluo-4 was excited with a 488 nm laser, and emission was detected between 505 and 605 nm. The sensitivity of the emission detector (photomultiplier tube, PMT) was chosen to avoiding pixel saturation, and was kept constant in all experiments. Images were taken every 2 s beginning 15 s before nsPEF exposure, and continued as a time series for 85 s after it. Images were quantified using ImageJ software [28]. A region of interest (ROI) was manually drawn around the perimeter of each cell, and the average pixel intensity was measured within it. For each cell, the emission at each time point was normalized to the mean of the first four images (before nsPEF delivery).

Typically, a mixed cluster of VGCC+ and VGCC− cells in the center of the field of vision was chosen for nsPEF exposure. Fluorescence of mCherry (Ex/em 543/640 nm) served as marker for the detection of transfected cells, but the expression of functional VGCC was always verified by measuring Ca^{2+} transients in response to depolarization by a brief superfusion with a modified physiological solution (with NaCl substituted for KCl). The response was quantified using ImageJ software, as described above, and cells were split into groups. Cells were considered VGCC− if their response stayed within ± 5% from the baseline. Cells were regarded VGCC+ if their response exceeded the baseline twofold or more (> 200%). Cells which did not fall into either