Bioelectrochemistry 100 (2014) 88–95

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Disassembly of actin structures by nanosecond pulsed electric field is a downstream effect of cell swelling

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ARTICLE INFO

Article history:
Received 5 August 2013
Received in revised form 27 December 2013
Accepted 14 January 2014
Available online 21 January 2014

Keywords:
Electric pulses
Nanopores
Plasma membrane
Electroporation
Cytoskeleton

ABSTRACT

Disruption of the actin cytoskeleton structures was reported as one of the characteristic effects of nanosecond-duration pulsed electric field (nsPEF) in both mammalian and plant cells. We utilized CHO cells that expressed the monomeric fluorescent protein (mApple) tagged to actin to test if nsPEF modifies the cell actin directly or as a consequence of cell membrane permeabilization. A train of four 600-ns pulses at 19.2 kV/cm (2 Hz) caused immediate cell membrane poration manifested by YO-PRO-1 dye uptake, gradual cell rounding and swelling. Concurrently, bright actin features were replaced by dimmer and uniform fluorescence of diffuse actin. To block the nsPEF-induced swelling, the bath buffer was isoosmotically supplemented with an electropore-impermeable solute (sucrose). A similar addition of a smaller, electropore-permeable solute (adonitol) served as a control. We demonstrated that sucrose efficiently blocked disassembly of actin features by nsPEF, whereas adonitol did not. Sucrose also attenuated bleaching of mApple-tagged actin in nsPEF-treated cells (as integrated over the cell volume), although did not fully prevent it. We conclude that disintegration of the actin cytoskeleton was a result of cell swelling, which, in turn, was caused by cell permeabilization by nsPEF and transmembrane diffusion of solutes which led to the osmotic imbalance.

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1. Introduction

Permeabilization of cells and tissues by nanosecond pulsed electric field (nsPEF) is a recent extension of conventional electroporation technology [1–4]. A distinguishing feature of nsPEF is the ability to produce intracellular effects with relatively minimum disruption of the plasma membrane, resulting in a wide range of bioeffects. At the cellular level, such effects include the enhancement of transmembrane transport [5,6], calcium mobilization [7–10], activation of phosphoinositide signaling [11] and MAPK pathways [12], phosphatidylserine externalization [13], mitochondrial membrane permeabilization [14], modulation of ion channels [15,16], cell swelling and blebbing [17], and necrotic and apoptotic cell death [1,18–21]. In principle, intense nsPEF treatments are likely to modify most cell functions; however, the chain of events from the primary nsPEF effect of membrane permeabilization to secondary and further downstream effects is poorly understood. It is also being debated if permeabilization of membranous structures is the only primary effect of nsPEF. For example, a recent study established ROS formation by nsPEF in both living cells and culture media [22], although its biological significance has yet to be explored.

In recent years, specific attention has been paid to the disruption of actin microfilaments by nsPEF. This attention, at least in part, is fueled by profound differences in nsPEF sensitivity across different cell types [18,23,24]. Differences in the cytoskeleton organization and the robustness of the cytoskeletal cortex underlying the cell membrane could potentially explain the different vulnerability to electroporation. Indeed, in plant cells, stabilization of actin increased the stability of the plasma membrane against electric permeabilization as recorded by a decreased penetration of Trypan Blue into the cytoplasm [25]. In mammalian cells, inhibition of actin polymerization with Latrunculin A significantly enhanced cell uptake of propidium and phosphatidylserine externalization [26]. Nonetheless, the authors concluded that differences in the cell stiffness could only partially be responsible for cell vulnerability to nsPEF. In a different study, disruption of the actin cytoskeleton in adherent cells prior to nsPEF exposure significantly reduced cell survival [27], but it is not clear whether the combined effect was specific to nsPEF or just reflected general "weakening" of cells by the actin-disrupting toxin. Disintegration of the cytoskeleton in the cell cortex, followed by contraction of actin filaments towards the nucleus in cells irreversibly permeabilized by nsPEF [28] and degradation of actin cytoskeleton in cells undergoing nsPEF-induced apoptosis [29] established a further

Abbreviations: DIC, differential interference contrast; nsPEF, nanosecond pulsed electric field; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.bioelechem.2014.01.004

link between actin and the nsPEF-induced cell death. In contrast to the above observations, exposure of U937 cells to very long, high-rate nsPEF trains (e.g., 2400 pulses at 20 Hz, 60 ns, 10 kV/cm) triggered rapid de novo actin cortex and scaffold formation within membrane blebs [30]; this phenomenon was observed only in a calcium-free bath buffer and led to the directional growth of giant longitudinal membrane protrusions capable of fast retraction after the exposure cessation.

It is now well established that intense nsPEF treatments permeabilize the cell plasma membrane to small solutes ("nanoelectroporation") [2,5,6,31–33]. These solutes travel down the electric and concentration gradients across the electroporated membrane, whereas larger solutes remain trapped inside the cell and create the osmotic force to attract water (the so-called colloid-osmotic mechanism) [17,32,34,35]. The increased pressure from the inside forces the cell to acquire a more spherical shape and to use membrane reserves for cell volume increase. One can hypothesize that cell rounding and swelling are exactly the reasons for disassembly of actin cytoskeleton, in order to accommodate the new cell shape. On the other hand, one cannot exclude that it is the disintegration of the cytoskeleton by nsPEF that weakens the cell adhesion, makes cells more spherical, and allows for their swelling, which eventually culminates in membrane rupture and cell death. To resolve these two pathways, we blocked the colloid-osmotic swelling in nsPEF treated cells and demonstrated that it also prevents cell rounding and cytoskeleton disintegration.

2. Materials and methods

2.1. Cell line

CHO-K1 cells (Chinese hamster ovary) were obtained from ATCC (Manassas, VA) and modified for stable expression of fluorescently tagged actin. The cells were transfected with a plasmid DNA encoding mApple-tagged human β-actin under a CMV promoter (a gift from Mike Davidson, Florida State University, Tallahassee, FL) using Effectene (Qiagen, Valencia, CA).

Cells stably expressing mApple-actin were selected with 400 μg/ml G418 (Calbiochem, Darmstadt, Germany), screened for fluorescence using an inverted microscope, and isolated with cloning rings. The cells were propagated in F12K medium (Mediatech Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 I.U./ml penicillin, 0.1 μg/ml streptomycin (CellGro), and 400 μg/ml G418. Cells were transferred onto poly-l-lysine covered glass coverslips 1–2 days prior to experiments. Transformed cells had the same appearance as the wild type CHO cells, but typically grew to a larger size.

2.2. Cell imaging and data analysis

A cover slip with cells was placed into a glass-bottomed chamber (Warner Instruments, Hamden, CT) mounted on an Olympus IX81 inverted microscope equipped with an FV 1000 confocal laser scanning system (Olympus America, Center Valley, PA). All experiments were performed at a room temperature of 22–24 °C. Fluorescent images of tagged actin (excitation: 543 nm; emission: 560–660 nm) were collected in parallel with differential-interference contrast (DIC) images using a 40× dry objective (NA 0.95). Although mApple emission is in the red spectrum, the fluorescent images were converted into a green pseudocolor for better clarity and visual contrast. For YO-PRO-1 dye uptake, we used a 488 nm laser and measured the emission in the 505–525 nm range.

In most experiments, we acquired either a time series of images of a selected X–Y plane, or Z-stacks of images at either 0.5 or 1 μm distance between the planes. For the time series, the images were taken every 10 s, and scanning for a single image took 5–7 s; three images were taken as a control prior to nsPEF delivery at 28 s, and up to 40 images after nsPEF. The time intervals indicated below are those between the onset of nsPEF train and the onset of a particular image scan.

In contrast, Z-stacks of images were obtained at a higher resolution and scanning took long time (4–7 min). Therefore, in each experiment one Z-stack was collected immediately prior to nsPEF exposure and another one at 10 min after it (sometimes followed by a third one at 20 min). 3D reconstruction of Z-stacks was accomplished with SlideBook 5.0 (Intelligent Imaging Innovations, Denver, CO).

Images were quantified with MetaMorph Advanced v. 7.7.10.0 (Molecular Devices, Foster City, CA) and the numerical data were further processed with Grapher 9.6 (Golden Software, Golden, CO). All numerical data in this paper are presented as mean values +/− s.e. for 7–13 cells in each group (from a minimum of 3 independent experiments). The statistical significance of differences between individual groups was estimated with a two-tailed *t*-test.

2.3. Chemicals and buffers

During experiments, the cells were kept in a physiological solution (hereinafter referred to as "saline") containing (in mM): 140 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.3, 300 mOsm/kg). For detection of cell membrane permeabilization by nsPEF, 1 μM of YO-PRO-1 dye was added to the saline. In order to inhibit the nsPEF-induced cell swelling, the saline was mixed 7:3 with an isoosmotic water solution of sucrose. Since such modification of the saline also decreased its electrical conductance from 1.53 to 1.08 S/m and it could change nsPEF effects, control samples were identically mixed with an isoosmotic adonitol solution. Saline with adonitol had the same conductance as saline with sucrose, but, due to smaller molecular size of adonitol, did not render protection against the colloid-osmotic swelling [17,19].

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for YO-PRO-1 (Invitrogen, Eugene, OR).

2.4. nsPEF exposure and dosimetry

The method of nsPEF exposure was the same as described earlier [5,6], with minor modifications. The electrode arrangement in relation to the cover slip and exposed cells was similar to that shown in the previous papers [36,37]. Nearly rectangular 600-ns pulses were generated in a transmission line-type circuit, by closing a MOSFET switch upon delivery of a TTL trigger pulse from pClamp software via a Digidata 1322A output (Molecular Devices). The same devices were employed to synchronize nsPEF delivery with image acquisition. NsPEF was delivered to a selected cell or a small group of cells on a glass coverslip with a pair of tungsten rod electrodes (100 μm diameter) driven by a robotic manipulator (MP-225, Sutter Instruments, Novato, CA). The major difference from the previous studies was a narrower gap between the nsPEF-delivering electrodes (100 μm) and a decreased height of the electrode tips above the coverslip (30 μm). These changes were intended to maximize the E-field at the cell location, albeit at the expense of field uniformity. The nsPEF rise time (10 to 90%) was 6.2 ns.

The E-field at the cell location was determined by 3D simulations with a finite element Maxwell equations solver Amaze 3D (Field Precision, Albuquerque, NM). At the maximum charging voltage to the transmission line (990 V), the E-field at the cell location was 20 kV/cm (+/−20%) for a single pulse and 19.2 kV/cm (+/−20%) for subsequent pulses when a train of pulses was delivered at 2 Hz. This difference was caused by a limitation in pulser charging time. For clarity, the small difference between the first and next pulses in trains was disregarded, and in this paper we refer to pulse trains as being at 19.2 kV/cm.

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