Biochimica et Biophysica Acta 1859 (2017) 438–445

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Nanosecond pulsed electric field induced dose dependent phosphatidylinositol-$_{4,5}$-bisphosphate signaling and intracellular electro-sensitization

Gleb P. Tolstykh [a,*], Melissa Tarango [a], Caleb C. Roth [b], Bennett L. Ibey [b]

[a] General Dynamics Information Technology, 4141 Petroleum Road, JBSA Fort Sam Houston, TX 78234, USA
[b] Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Bioeffects Division, Radio Frequency Bioeffects Branch, 4141 Petroleum Road, JBSA Fort Sam Houston, TX 78234, USA

ARTICLE INFO

Article history:
Received 6 September 2016
Received in revised form 15 December 2016
Accepted 2 January 2017
Available online 05 January 2017

Keywords:
Nanosecond electric pulses
PIP_2 depletion
Diacylglycerol
Inositol-$_{1,4,5}$-trisphosphate
Nanoporation
Membrane

ABSTRACT

Previously, it was demonstrated that nanometer-sized pores (nanopores) are formed in outer cellular membranes after exposure to nanosecond electric pulses (nsEPs). We reported that plasma membrane nanoporation affects phospholipids of the cell membrane, culminating in cascading phosphoinositide phosphatidylinositol-$_{4,5}$-bisphosphate (PIP_2) intracellular signaling. In the current study, we show that nsEPs initiated electric field (EF) dose-dependent PIP_2 hydrolysis and/or depletion from the plasma membrane. This process was confirmed using fluorescent optical probes of PIP_2 hydrolysis: PLCδ-PH-EGFP and GFP-C1-PKCγ-C_{1a}. The 50% maximum response occurs with a single 600 ns pulse achieving an effective dose (ED_{50}) of EF ~ 8 kV/cm within our model cell system. At 16.2 kV/cm, the ED_{50} for the pulse width was 484 ns. Reduction of the pulse width or EF amplitude gradually reduced the observed effect, but twenty 60 ns 16.2 kV/cm pulses produced an effect similar to a single 600 ns pulse of the same amplitude. Propidium iodide (PI) uptake after the nsEP exposure confirmed a strong relationship between EF-induced plasma membrane impact and PIP_2 depletion. These results have expanded our current knowledge of nsEPs dependent cell physiological effects, and serve as a basis for model development of new exposure standards, providing novel tools for drug independent stimulation and approaches to differential modulation of key cellular functions.

1. Introduction

The application of high magnitude, ultrashort (<1 μs) electric pulses (EPs) to cells produces diverse effects, including neuronal and cardiomyocytes excitation, stimulation of cellular autophagy, nuclear granulation, intracellular calcium bursts, cytoskeletal changes, blebbing, swelling, and initiation of apoptotic cell death [1–12]. These post-exposure changes in intracellular Ca^{2+} concentration start immediately after nanosecond electric pulses (nsEPs) exposure and last for minutes.

The aforementioned biological effects of nsEPs exposure have been largely attributed to plasma membrane nanoporation or permeabilization of intracellular organelles [13,14]. Long-lasting nanoporation after nsEPs exposure was confirmed using imaging and electrophysiological techniques. The recovery of plasma membrane conductance due to putative pore resealing took several minutes, and the nanopores had complex conductive properties similar to "classic" protein ion channels [15–17]. However, in contrast to this notion, molecular dynamics simulations predict the life span of the nanopores to be <100 ns [18]. Thus, it is unlikely that nanoporation is solely responsible for the long lasting bioeffects after nsEPs exposure. Rather, plasma membrane nanoporation could be the initial step in a complex chain of reactions leading to deregulation of membrane transport proteins and prolonged post-exposure intracellular physiological effects. We hypothesize that nsEPs acts directly on the plasma membrane initiated by a complex chain of biochemical events resulting in the observed, but diverse cellular responses.

Numerous membrane transport proteins are regulated by the concentration of phosphoinositides in the plasma membrane [19,20]. Specifically, phosphatidylinositol 4,5-bisphosphate (PIP_2) on the internal layer of the plasma membrane plays an important role as a regulator of ion channels, a source of second messenger compounds, and as an anchor for cytoskeletal elements [21–24]. The function of several ion channels and transporters is dependent on the presence of PIP_2 in the plasma membrane and others are regulated by intracellular signaling utilizing components of PIP_2 hydrolysis [25]. Stimulation of $G_{q/11}$ coupled receptors initiates an intracellular phosphoinositide signaling cascade, leading to activation of phospholipase C (PLC), and results in the hydrolytic production of diacylglycerol (DAG) and inositol-$_{1,4,5}$-trisphosphate (IP_3). It has been suggested that after PIP_2 hydrolysis, the resultant elevated cytosolic Ca^{2+} from IP_3-sensitive Ca^{2+} stores activates protein kinase C (PKC). The newly available DAG in the plasma membrane

* Corresponding author.
E-mail address: gleb.tolstykh.ctr@us.af.mil (G.P. Tolstykh).

http://dx.doi.org/10.1016/j.bbamem.2017.01.003
0005-2736/

attracts activated PKC, which then triggers a host of other cellular responses [21,24,26–29].

Recently, we demonstrated that exposure of cells to a single 16.2 kV/cm 600 nsEP triggers phosphoinositide signaling similar to stimulation of the $G_{q/11}$-coupled receptors [30]. However, we observed the same results after nsEP exposure using CHO-K1 cells without specific G_q-coupled receptors, outlining the possibility of direct nsEP impact on the plasma membrane [31]. Correspondingly, we have shown that exposure of cells to 600 nsEP directly initiates PIP_2 depletion from the internal side of the plasma membrane, even in the absence of PLC activity (when blocked with edelfosine) [32,33]. Recent Ca^{2+} imaging experiments, where nsEP exposure triggered IP_3-induced Ca^{2+} release in edelfosine treated cells and in the absence of Ca^{2+} in the external media, support the possibility of a direct effect on the plasma membrane [34]. Accordingly, the addition of Ca^{2+} to external media dramatically accelerates both nsEP and drug-induced PIP_2 hydrolysis due to enhanced PLC activity [27,32].

Other evidence of a direct nsEP impact includes data suggesting that, in a Ca^{2+}-free environment, DAG production only occurs on the side of the cell facing the anodic electrode [32]. Several additional noteworthy results of these studies deserve careful attention. The post-nsEP exposure PIP_2 recovery back to the plasma membrane took ~10 min, and cells with drug-induced PIP_2 hydrolysis demonstrate cellular swelling and blebbing during PIP_2 dissociation to IP_3 and DAG [33]. The known regulatory role of PIP_2 in docking of the cytoskeleton to the plasma membrane [35] could be one of the key components of the long lasting effects after nsEP exposure. The PIP_2, DAG, PKC, and PLC enhance store operated Ca^{2+} entry [36], open and sensitize some nonspecific cation ion channels [37], including store operated and mechanosensitive TRP channels [38–41]. Thus, nsEP dependent nanoporation in concert with nsEP-induced intracellular signaling, specifically hydrolysis of PIP_2, could activate multiple downstream events explaining the long lasting multifarious effects observed previously.

From our previous data, we believe that PIP_2 plays an integral role in the cellular response to nsEP and underpins much of what has been observed by our research group and others in terms of the multifarious effects of exposure. To further this concept the current study aims to determine the ED_{50} (the 50% of maximum response) for nsEP-induced PIP_2 depletion. We measured this effect by using confocal microscopy to monitor the translocation of optical probes of PIP_2 hydrolysis with variable nsEP parameters. In addition to advancing the current knowledge of nsEP biological mechanisms, the results of the current study could aid in developing the ability to control or manipulate concentration of PIP_2 in the internal leaflet of the plasma membrane by nsEP. While use of μsEPs and nsEPs has already been proposed for cancer therapy [42], this application would enable a noninvasive and drug-free method to regulate physiological homeostasis at the cellular level.

2. Methods

2.1. Cell culture

For the experiments, CHO-K1 cells were transfected to stably express human muscarinic type 1 (hM_1) receptor. These cells were kindly provided by Dr. Mark S. Shapiro (University of Texas Health Science Center at San Antonio) [43]. CHO-hM_1 cells were selected to provide continuity with previous experiments, as well as to allow for potential drug treatment with agonists of the hM_1 receptor for positive control experiments. Cells were cultured in standard complete growth medium consisting of Ham's F-12K media (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic (Life Technologies), and 0.48% G418 antibiotic (Life Technologies) to ensure transfection stability. Cells were plated for imaging on 35 mm glass-bottomed dishes (MatTek No. 0, Ashland, MA) coated with D-lysine approximately 18 h prior to staining and exposure, and incubated in complete growth media overnight at 37 °C.

2.2. Pulsing system and general set up

In this study a single monopolar (MP), bipolar (BP) and multiple MP nsEPs were used as described in previous publications [4,30–34,44]. Briefly, CHO-hM1 cells were exposed to a single MP, BP or multiple MP (delivered at 5 Hz) EPs of varying duration (10, 30, 60, 200, 400, or 600 ns) using a custom microelectrode comprised of a pair of cylindrical 125 μm tungsten wires placed in parallel with an edge to edge separation of 150 μm. The electrode was positioned 50 μm above the glass surface by a micromanipulator (Sutter MP285) directly above a target cell or cell population. The resulting EF amplitudes for MP pulses were 2 - 16.2 kV/cm, and 28 kV/cm (peak to peak) for a single BP pulse. Different EFs for MP exposures were achieved by lowering charging voltage feeding the pulser (1, 0.75, 0.5, 0.125 and 0-sham kV). To precisely deliver the exposure while imaging the cell, a Stanford DG535 digital delay generator was programmed to trigger the Zeiss LSM-710 confocal microscope to begin image acquisition using Zen 9.0 (Carl Zeiss MicroImaging) imaging software. Next, after a 5-s delay (chosen to measure baseline fluorescence), a second signal was sent to a Hewlett-Packard (HP) 8112A pulse generator to trigger the MP nsEP. We used a custom-made BP pulse generator with a full-bridge voltage source inverter (VSI) consisting of four metal-oxide-semiconductor field-effect transistors (MOSFETs) (IXYS, IXFB38N100Q2, 1 kV, Milpitas, CA) for the BP nsEPs. Images were acquired at a rate of one 40× image per second (512 × 512) for 180 s (360 total images – 180 T-PMT for bright field and 180 FL1 for GFP fluorescence). To ensure accurate pulse delivery, both the pulse amplitude and width were monitored on a Tektronix TDS3052 500-MHz oscilloscope using a 100× voltage probe (Tektronix, P5100A) placed across a 50 Ω resistor in parallel with the electrodes. Due to differences between MP and BP pulsers, the resultant single phase pulse amplitude for a 1 kV charging voltage was ~400 V for the MP and ~700 V for the BP pulses, resulting in higher EF amplitude for BP nsEPs.

2.3. Probe of PIP_2 hydrolysis and transfection

To monitor nsPEF-induced PIP_2 hydrolysis, we used PLCδ-PH-EGFP (Addgene plasmid 21179) and GFP-C_1-PKCγ-C_{1a} (Addgene plasmid 21179) constructs originally developed in the Tobias Meyer laboratory [45,46]. PLCδ-PH-EGFP is a human PLCδ, which binds to PIP_2 and IP_3 through its pleckstrin homology (PH) domain. This probe is fused with EGFP for visualization in the cell. In cells before stimulation, where the level of PIP_2 in the membrane is high and intracellular level of IP_3 is low, the probe is bound to PIP_2 and localized in the membrane. During PIP_2 hydrolysis, the probe rapidly translocates with bonded IP_3 molecules to the cytoplasm. PIP_2 resynthesis by phosphatidylinositol-specific kinases recovers the plasma membrane fluorescence. GFP-C_1-PKCγ-C_{1a} is the C_1 (Cys) domain of protein kinase Cγ (PKCγ) tagged with GFP. This probe is a reporter of DAG production, as it translocates from the cytoplasm to the membrane upon increases in membrane DAG following PIP_2 hydrolysis. Probes were transiently transfected into cultured CHO-hM_1 cells by Lonza Nucleofector™2d device using standard protocol. The percentage of cells expressing the GFP-constructs after such transfection was approximately 35–40%. All experiments reported in this paper were performed at room temperature (24–26 °C).

2.4. Solutions

Cells were exposed in a Ca^{2+}-rich solution consisting of 2 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, and 135 mM NaCl with a pH of 7.4 and osmolarity of 290–310 mOsm.