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nsPEF-induced PIP_2 depletion, PLC activity and actin cytoskeletal cortex remodeling are responsible for post-exposure cellular swelling and blebbing

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ABSTRACT

Cell swelling and blebbing has been commonly observed following nanosecond pulsed electric field (nsPEF) exposure. The hypothesized origin of these effects is nanoporation of the plasma membrane (PM) followed by transmembrane diffusion of extracellular fluid and disassembly of cortical actin structures. This investigation will provide evidence that shows passive movement of fluid into the cell through nanopores and increase of intracellular osmotic pressure are not solely responsible for this observed phenomena. We demonstrate that phosphatidylinositol-4,5-bisphosphate (PIP_2) depletion and hydrolysis are critical steps in the chain reaction leading to cellular blebbing and swelling. PIP_2 is heavily involved in osmoregulation by modulation of ion channels and also serves as an intracellular membrane anchor to cortical actin and phospholipase C (PLC). Given the rather critical role that PIP_2 depletion appears to play in the response of cells to nsPEF exposure, it remains unclear how its downstream effects and, specifically, ion channel regulation may contribute to cellular swelling, blebbing, and unknown mechanisms of the lasting "permeabilization" of the PM.

1. Introduction

Exposure to nanosecond pulsed electrical fields (nsPEF) results in a myriad of observable cellular effects, including alteration of intracellular Ca^{2+} homeostasis, nuclear granulation, cytoskeletal changes, cellular blebbing, swelling, and initiation of apoptotic cell death [1–8]. While these effects are often attributed to the direct nanoporation of both the plasma and organelle membranes [9–11], the underlying mechanisms are not well understood.

Some of these nsPEF-induced biological effects appear to be similar to situations observed during the normal lifespan of mammalian cells. For example, plasma membrane (PM) blebs occur during cytokinesis, cell migration, proliferation, and apoptosis [12]. It has been shown that both PIP_2 and phosphoinositide-specific PLC are required for regulation of cortical actin dynamics during cytokinesis, since PIP_2 needs to be continuously hydrolyzed for successful completion of cell division, and its hydrolysis pair diacylglycerol (DAG) with protein kinase C (PKC) to stimulate cellular growth and proliferation [13,14]. Likewise, PIP_2 and phosphoinositide-specific PLC are heavily involved in regulation of vital cellular functions such as modulation of PM transport proteins, cytoskeleton dynamics, intracellular Ca^{2+} homeostasis, and regulation of cellular volume [15–21]. While alterations of these functions have all been observed after nsPEF exposures, their relation to the nsPEF-induced PIP_2 signaling pathway is not understood.

Recently, we confirmed that a single 16.2 kV/cm, 600 ns electric pulse initiates an intracellular phosphoinositide PIP_2 signaling cascade similar to one initiated by activation of $G_{q/11}$-coupled receptors, but in cells without such receptors [22]. PIP_2 depletion by nsPEF exposure was demonstrated by direct monitoring of translocation of optical probes of PIP_2 hydrolysis. The nsPEF-induced PIP_2 signaling mirrored the responses following human muscarinic acetylcholine $G_{q/11}$-coupled receptor (hM_1) activation [22,23]. Additionally, the nsPEF-induced increase in intracellular Ca^{2+} is almost identical to the calcium rise observed after purinergic P_2Y_6 $G_{q/11}$-coupled receptor stimulation [24], further suggesting PLC activation and induction of PIP_2 signaling. Initiation of PIP_2 signaling results in production of DAG and inositol-1,4,5-trisphosphate (IP_3) [25], leading to elevation of cytosolic Ca^{2+} from IP_3-sensitive Ca^{2+} stores and DAG – dependent activation of PKC

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[26].

Permeabilization of the PM by nsPEF is an important step in initiation of the response, but cannot be solely accounted for the long-lasting effects due to the theoretically predicted short lifespan (~100 ns) of nanopores in biomembranes [27]. Cell swelling and blebbing occurs seconds after nsPEF exposure and lasts for minutes, comparable to the time needed for PIP_2 recovery back to the PM [22,23]. Since PIP_2 is known to anchor cortical actin [28] and PLC-dependent PIP_2 hydrolysis modulates cytoplasmic cell volume regulatory ion channels [29,30], we hypothesize that nsPEF-stimulated PLC activity and resulting PIP_2 depletion are primarily responsible for the post-exposure, physiological effects of actin cortex remodeling, cellular swelling, and blebbing. This work aims to further expand our previous findings that disintegration of the cortical actin structures is related to cellular swelling initiated by transmembrane diffusion of water after nsPEF-induced PM nanoporation [7].

2. Methods

2.1. Nanosecond pulse exposure

Single and multiple (20 at 5 Hz rate) nsPEFs were delivered to cells using a pair of 125 μm-diameter tungsten electrodes spaced 120 μm apart as detailed in previous publications [1,4,22,23]. Finite difference time domain (FDTD) modeling was used to model the exposure system, and FDTD modeling predicted a 16.2 kV/cm peak field for a 1 kV charging voltage (0.5 kV nsPEF amplitude). To synchronize image acquisition and pulse delivery, a Stanford DG535 digital delay generator was programmed to trigger the Zeiss LSM-710 confocal microscope to begin image acquisition. After a 5-s preset delay, a HP 8112 A pulse generator delivered a specific number of nsPEFs.

2.2. Probe of PIP_2 hydrolysis

PLCδ-PH-EGFP DNA construct (Addgene plasmid 21179) [22,23] was transiently transfected into the Chinese hamster ovarian (CHO) cells already stably expressing m-Apple-actin or G_{q11}-coupled hM_1 and angiotensin II (AngII) receptors using a Lonza 2D Nucleofector™ device. Dr. Mark S. Shapiro (Department of Physiology, University of Texas Health Science Center at San Antonio) kindly provided the hM_1 and AngII cells lines [31], and the m-Apple-actin cell line was created in-house as previously described [7].

2.3. Experimental procedures

The m-Apple-actin, hM_1, and AngII receptor stable cell cultures used a standard complete growth medium consisting of Ham's F-12K media supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic, and 0.48% G418 to ensure transfection stability. Cells were plated on poly-L-lysine coated 35 mm glass coverslip bottom dishes (MatTek No. 0, Ashland, MA) for 24 h prior to experimentation. Cells were rinsed to remove any residual growth medium, and 2 mL of a standard buffer solution (pH 7.4, 290–310 mOsm) that consisted of 2 mM $MgCl_2$, 5 mM KCL, 10 mM HEPES, 10 mM Glucose, 2 mM $CaCl_2$, and 135 mM NaCl was added. In some experiments, the $CaCl_2$ was replaced with 2 mM K-EGTA to create Ca^{2+}- free external buffer.

For intracellular Ca^{2+} measurement, CHO-hM_1 cells were loaded with Calcium Green-1 AM ester (CaGr). A 2 μL aliquot of 3 mM CaGr stock was added to 2 mL of standard buffer, and administered to cells incubated at 37 °C for 30 min. After 30 min, the loading buffer was replaced with standard buffer and images were collected using an inverted Zeiss 710 LSM confocal microscope equipped with a 40X (1.3 N.A.) oil immersion objective.

To compare nsPEF effects with well-known effects caused by endogenous PLC activation, some experiments were paired with $G_{q/11}$-coupled hM1 receptor agonist oxotremorine (OxoM, 10 μM) or AngII receptor agonist angiotensin II (AngII, 10 μM). In a subset of experiments, cells were incubated with PLC blocker edelfosine (10–20 μM) for 30 min prior to OxoM, AngII, or nsPEF exposure. Agonists were added by circulating fresh bath buffer containing either OxoM or AngII at a flow rate of 2 mL/min. All media, chemicals and pharmaceuticals were obtained from Life Technologies, Tocris Bioscience or Sigma-Aldrich. Also, 4 mM propidium iodide (PI) (BD Bioscience) was added to the external solution during some experiments to verify cell viability.

2.4. Data analysis

Cell fluorescence was measured using ImageJ software (NIH) [32]. To measure CaGr fluorescence changes, regions of interest (ROI) were drawn in the cytoplasmic region of cells under study. For PLCδ-PH-EGFP and m-Apple-actin fluorescence measurements, ROI were carefully drawn to demark the PM and the cytoplasm of the cell. We used slightly wider ROI during PM measurements to compensate for PM moving during blebbing. Mean fluorescence was measured for each ROI for all images using ImageJ Multi-Measure. Similarly, to measure cell perimeter lengths, ROI were hand-drawn around each cell, and the perimeter was measured. These values were transferred to GraphPad Prism 6 software for statistical analysis and plotting. The responses were calculated for each cell as a percentage difference (ΔF, %) from the mean of the four frames prior to exposure (Baseline) to the frames taken after exposure (Value) using the formula: 100×(Value – Baseline)/Baseline. All imaging experiments were performed at one frame per second (1 Hz).

3. Results and discussion

The roles of local cytoskeleton and changes of intracellular hydrostatic pressure in blebbing cells after nsPEF exposure [7] have been studied previously. However, the role that PIP_2 depletion and hydrolysis play in the nsPEF-induced cellular response has not been evaluated. To achieve this goal, we used edelfosine (ET-18-O-CH3), a synthetic alkyl-lysophospholipid that blocks PLC to prevent PIP_2 hydrolysis [13,33]. Since edelfosine could arrest cell cycle, inhibit cytokinesis, and initiate apoptosis [13,34–36], we performed a series of experiments to determine its safe operational dose. A small fraction of cells (2%) bathed for 20 min in the 10 μM edelfosine containing buffer experienced PI uptake. However, 100% of the cells had PI uptake after the dose of edelfosine was increased above 30 μM (data not shown). PI uptake is a widely accepted sign of attenuation of cellular viability and PM integrity [37].

To verify the potency of edelfosine as a blocker of PLC activity, we monitored intracellular Ca^{2+} release after $G_{q/11}$-coupled receptor activation. By using Ca^{2+}-chelation in outside buffer, we determined that a 20–30 min pretreatment of cells in 10 μM edelfosine resulted in sufficient decrease of PLC-dependent intracellular Ca^{2+} release from the ER (Fig. 1 (A)). In contrast, but similar to our previous observations [23], addition of 10–20 μM edelfosine was unable to completely prevent intracellular Ca^{2+} spikes after a single 600 ns, 16.2 kV/cm electric pulse (EP) (Fig. 1 (B)). The persistence of the intracellular Ca^{2+} rise suggests that the initial nsPEF-induced PIP_2 depletion is a result of strong, direct mechanical impact on the cellular PM, and not PLC-mediated hydrolysis.

Based on these observations, we chose to use 20 μM of edelfosine to maximally block PLC activity. To outline the critical role of PLC in the cell response to nsPEF, all edelfosine experiments were performed in Ca^{2+} containing external buffer. Fig. 2 shows translocation of the PLCδ-PH-EGFP construct from the PM to the cytoplasm after $G_{q/11}$-coupled receptor stimulation by agonists. This stimulation leads to activation of PLC, which then hydrolyzes PIP_2 to IP_3 and DAG. The PLCδ-PH-EGFP construct, with tagged IP_3, accumulates in the cytoplasm – directly