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Gadolinium blocks membrane permeabilization induced by nanosecond electric pulses and reduces cell death

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ABSTRACT

It has been widely accepted that nanosecond electric pulses (nsEP) are distinguished from micro- and millisecond duration pulses by their ability to cause intracellular effects and cell death with reduced effects on the cell plasma membrane. However, we found that nsEP-induced cell death is most likely mediated by the plasma membrane disruption. We showed that nsEP can cause long-lasting (minutes) increase in plasma membrane electrical conductance and disrupt electrolyte balance, followed by water uptake, cell swelling and blebbing. These effects of plasma membrane permeabilization could be blocked by Gd^{3+} in a dose-dependent manner, with a threshold at sub-micromolar concentrations. Consequently, Gd^{3+} protected cells from nsEP-induced cell death, thereby pointing to plasma membrane permeabilization as a likely primary mechanism of lethal cell damage.

1. Introduction

Tissue ablation and tumor destruction are probably the most promising medical applications of high-voltage nsEP. This treatment induced apoptotic and necrotic death in various cancer cells in vitro, stimulated tumor regression in vivo, and was successfully employed to treat basal cell carcinoma in a human trial [1–3]. However, the primary target and cell mechanisms responsible for nsEP-induced cell death are not known, which hinders translation of this modality into clinical practice.

In early studies, nsEP were shown to have limited effects on the plasma membrane and target intracellular structures directly [4–7]. Indeed, dielectric breakdown of the plasma membrane, which is the best known effect of longer (micro- and millisecond range) electric pulses [8], was ruled out by the lack of immediate uptake of membrane integrity marker dyes [1,6,9] and nsEP-induced apoptosis was supposed to be independent from plasma membrane electroporation [1]. However, several publications showed that nsEP can charge plasma membrane and change the transmembrane potential [10,11], cause fast externalization of phosphatidylserine residues [9,12], induce plasma membrane permeabilization [11,13,14] and calcium influx through the plasma membrane [15].

Indeed, as predicted by electrical models and simulations, nsEP have effects on both intracellular structures and plasma membrane. Reducing the pulses duration results in decreased plasma membrane permeabilization and delayed propidium uptake with increased intracellular effects [1,13]. On the contrary increasing the pulse duration, the number of pulses or the electric field intensity results in faster uptake of membrane permeability markers and increases the percentage of permeabilized cells [11,16].

Nonetheless, recently it was reported that even a single 60 ns pulse at 12 kV/cm was enough to cause a profound and long-lasting (minutes) increase in membrane electrical conductance, accompanied by the loss of the membrane potential and permeabilization to small ions, but not to larger molecules such as propidium [17,18]. Likewise, simulation studies found that nsEP should open nanometer-size electropores both in the cell plasma membrane and organelles [19,20], and the existence of stable nanopores in the plasma membrane of nsEP-treated cells has been confirmed experimentally [21]. Furthermore, membrane permeabilization to small ions occurred at nsEP intensities significantly lower than the threshold for other known nsEP effects [22]. Therefore it is likely that plasma membrane permeabilization was missed by earlier studies due to the limits of the detection techniques used. Thereby suggesting that it could be a primary mechanism of other nsEP effects, including nsEP-induced cell death.

Should it be the case, then one might reasonably anticipate that chemical and physical agents that inhibit nsEP-induced plasma membrane permeabilization effect will also increase the survival of nsEP-treated cells. So far, among various chemical agents tested, only the lanthanide ions (Gd^{3+} and La^{3+}) were found to inhibit the nsEP-induced permeabilization, and, most likely, it was a direct effect on the membrane [17]. The lanthanides also prevented swelling and blebbing in severely exposed cells, which supported the idea that these necrotic manifestations could be caused by membrane disruption. However, potential effect of the lanthanides on cell survival after nsEP

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exposure has not been studied. Furthermore, lanthanides were only tested at high (millimolar) concentrations, which increased the likelihood of non-specific mechanisms of their action.

The goals of the present study were (1) to explore modulation of membrane effects of nsEP by Gd^{3+} in a wide range of concentrations, and (2) to test if Gd^{3+} can also increase cell survival after nsEP exposure. If inhibition of nsEP-induced membrane permeabilization by Gd^{3+} also improves cell survival, this observation would point at the impairment of the plasma membrane barrier function as a likely primary mechanism responsible for nsEP-induced cell death.

2. Materials and methods

2.1. Cell cultures

Jurkat clone E6-1 (human T cell leukemia) and GH3 cells (murine pituitary) were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in the logarithmic stage of growth at 37 °C with 5% CO_2 in air and used at passages 3 to 15. GH3 cells were cultured in Ham's F12K medium supplemented with 2.5% fetal bovine serum (FBS) and 15% horse serum. Jurkat cells were propagated in RPMI-1640 medium with 10% FBS and 2 mM L-glutamine. The growth media also contained 100 IU/ml penicillin/streptomycin. The media and its components were purchased from Mediatech Cellgro (Herndon, VA) except for the animal sera (Atlanta Biologicals, Norcross, GA). For patch clamp and imaging experiments, cells were allowed a minimum of one day to adhere to glass coverslips covered with poly-L-lysine (Sigma-Aldrich, St. Louis, MO).

2.2. Chemicals and solutions

Cells were exposed to nsEP in a buffer composed of (hereinafter in mM) 135 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, *X* $GdCl_3$, and 10 glucose (pH 7.4), where *X* was varied from 0 to 1 mM. To assess plasma membrane integrity by dye exclusion, this buffer was supplemented with a membrane-impermeable fluorescent DNA stain, propidium iodide (PI, 10 or 30 μg/ml). For patch clamp experiments, recording pipette was filled with 140 KCl, 5 K-EGTA, 4 $MgCl_2$, and 10 HEPES (pH 7.2). The osmolality of the solutions was between 290 and 310 mOsm, as measured with a freezing point microosmometer (Advanced Instruments, Inc., Norwood, MA, USA). All chemicals were purchased from Sigma-Aldrich.

2.3. Cell imaging

Cells on a coverslip were transferred into a glass-bottomed chamber (Warner Instruments, Hamden, CT) mounted on an IX71 microscope configured with an FluoView 300 confocal laser scanning system (Olympus America Inc., Center Valley, PA). Differential interference contrast (DIC) and fluorescence images of cells were obtained with a 40×, 0.75 NA no-immersion objective. The images were taken as a time series, starting before nsEP exposure and continuing for several minutes after it. The effect of Gd^{3+} was identified by comparing morphological changes and propidium uptake in cells that were held at different Gd^{3+} concentrations while receiving the same nsEP treatments. Cell images were quantified off-line with MetaMorph v. 7.5 (MDS, Foster City, CA).

2.4. Patch clamp

We used the same setup as for the cell imaging, but the chamber was continually superfused with Gd^{3+}-free bath buffer at a rate of 0.9 ml/min. Unlike the imaging experiments, Gd^{3+}-containing buffer was applied 40–50 s after nsEP exposure, at 0.15 ml/min locally to the selected cell using 28G MicroFil needle (World Precision Instruments, Sarasota, FL). These experiments were intended to check if Gd^{3+} can revert nsEP-induced changes in the plasma membrane electrical conductance.

Recording pipettes were pulled from borosilicate glass (BF150-86-10, Sutter Instrument, Novato, CA) to a tip resistance of 1.5–3 MΩ. Seal and access resistance values were between 2 and 10 GΩ and 10 and 20 MΩ, respectively. Whole-cell currents were probed every 5–10 s by applying 120 ms voltage steps in 10 mV increments, from −100 to +40 mV; holding potential between the sweeps was −80 mV. Currents were measured as mean value at the plateau level, 50–110 ms after the start of the step. Passive membrane resistance (R_m) was defined as $\Delta V/\Delta I$ in a range that contained no visible voltage-activated currents, from −80 to −70 mV.

The data were collected using a Multiclamp 700B amplifier, Digidata 1322A A-D converter, and pCLAMP 10 software (MDS). Command voltages were corrected for the junction potential of −4.2 mV.

2.5. NsEP exposure

Two different systems were employed for exposure of individual cells on coverslips (patch clamp and microscopy measurements) and for bulk exposure of cell population in electroporation cuvettes (cell survival studies).

The setup for individual cell exposure was previously described [21]. In brief, 60 or 600 ns pulses were generated in a transmission line-type circuit and delivered to a selected cell with a pair of tungsten rod electrodes (0.1 mm diameter, 0.16 mm gap). These electrodes were positioned precisely 50 μm above the coverslip surface so that the selected cell was in the middle of the gap between their tips. The E-field at the cell location between the electrodes was determined by 3D simulations with a finite element Maxwell equations solver Amaze 3D (Field Precision, Albuquerque, NM).

For cell survival studies, 2×10^6 cells suspended in 420 μl of bath buffer (with or without Gd^{3+}) were placed in a 2 mm gap electroporation cuvette (Biosmith Biotech, San Diego, CA). 60 ns pulses at 2 Hz were produced by a Blumlein line generator; the E-field in the cuvette was calculated by dividing the applied voltage by the gap distance. Five minutes after exposure, cells were diluted 5× by the same buffer that was used for exposure and incubated for 0.25 to 5 h until cell viability measurements. All nsEP exposures and subsequent manipulations (except MTT assay, see below) were performed at a room temperature of 22–24 °C.

2.6. Cell viability assays

Propidium exclusion, as measured by flow cytometry 0.25 to 5 h post nsEP exposure, served as a principal index of cell survival. PI was added to an aliquot of exposed cells 5 min prior to measurements to a final concentration of 10 μg/ml. A total of 10,000 cells per sample were checked for propidium uptake using a FACSARIA flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). Cells that showed no propidium fluorescence (488 nm excitation, 695/40 nm emission) were defined as viable. The number of viable cells in each group was expressed as a percentage to the respective parallel control sample (sham exposure and no Gd^{3+}).

Of note, electroporation of cells by nsEP could cause modest immediate uptake of propidium (see fluorescent microscopy data below), which did not necessarily indicate cell death (the membrane could potentially re-seal later on [21]. In contrast, intense propidium uptake at long time intervals post exposure was regarded as permanent membrane damage and cell death.

In addition, cell viability was assessed by the activity of mitochondrial dehydrogenases, as measured by conversion of MTT reagent into blue formazan (CellQuanti-MTT cell viability assay kit, BioAssay Systems, Hayward, CA). One hour after exposure, 15 μl of MTT reagent and PI (to 10 μg/ml) were added to 100 μl aliquots of cell suspension