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Multiple nanosecond electric pulses increase the number but not the size of long-lived nanopores in the cell membrane

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ABSTRACT

Exposure to intense, nanosecond-duration electric pulses (nsEP) opens small but long-lived pores in the plasma membrane. We quantified the cell uptake of two membrane integrity marker dyes, YO-PRO-1 (YP) and propidium (Pr) in order to test whether the pore size is affected by the number of nsEP. The fluorescence of the dyes was calibrated against their concentrations by confocal imaging of stained homogenates of the cells. The calibrations revealed a two-phase dependence of Pr emission on the concentration (with a slower rise at < 4 μM) and a linear dependence for YP. CHO cells were exposed to nsEP trains (1 to 100 pulses, 60 ns, 13.2 kV/cm, 10 Hz) with Pr and YP in the medium, and the uptake of the dyes was monitored by time-lapse imaging for 3 min. Even a single nsEP triggered a modest but detectable entry of both dyes, which increased linearly when more pulses were applied. The influx of Pr per pulse was constant and independent of the pulse number. The influx of YP per pulse was highest with 1- and 2-pulse exposures, decreasing to about twice the Pr level for trains from 5 to 100 pulses. The constant YP/Pr influx ratio for trains of 5 to 100 pulses suggests that increasing the number of pulses permeabilizes cells to a greater extent by increasing the pore number and not the pore diameter.

1. Introduction

Uptake of propidium (Pr) by cells exposed to intense, pulsed electric fields is among the most common methods for revealing cell membrane permeabilization [1–16]. This dye is essentially non-fluorescent in the extracellular medium, but the emission increases 20- to 30-fold upon entering cells and binding to nucleic acids [17]. In contrast to conventional electroporation with milli- and microsecond electric pulses, early studies with nanosecond electric pulses (nsEP) reported diverse cellular effects (calcium activation, apoptosis, swelling, and phosphatidylserine externalization) that occurred in the absence of detectable Pr uptake [18–23] or only with a delayed (secondary) Pr uptake [18,23,24]. These results were interpreted as a proof of a direct intracellular impact of nsEP, consistent with theoretical predictions [1, 22,25,26]. However, later studies demonstrated permeabilization of the cell membrane exposed to nsEP by measuring its electrical conductance [10,15,27,28], the uptake of Tl^+ [6,17], Ca^{2+} [29–31], water [4,10], and YO-PRO-1 (YP) dye (which is smaller than Pr) [21, 32]. These data suggested that pores formed by nsEP are permeable to smaller solutes but not to Pr. Hence, nsEP-opened pores were thought to be smaller than about 1.5 nm, which is the estimated minimum diameter for the passage of Pr^{2+} cation [6]. At the same time, a recent study reported permeabilization of cells to bleomycin, a molecule larger than Pr, by 10-ns pulses at 40 kV/cm [33].

An independent and arguably more accurate approach for estimation of the pore size is based on the blocking of colloid-osmotic cell swelling [4,34]. In brief, only small, pore-permeable solutes leak out of electroporated cells, whereas larger molecules remain trapped in the cell. Concurrently, small solutes from the extracellular medium enter the cell down the electric and/or concentration gradients. The osmotic pressure inside (small solutes plus trapped large solutes) exceeds the outside pressure, leading to water uptake and cell swelling. This process can be blocked, and swelling turned into shrinking, by replacing small solutes outside the cell with larger ones which do not pass into the cell through nanopores. "Titrating" the blocking of cell swelling with sugars and polyethylene glycols of different sizes has set the average diameter of pores opened by 60- and 600-ns pulses at 0.9–1.3 nm [4], consistent with dye uptake findings. Finally, recent *in silico* modeling of nsEP effects predicts the formation of large numbers of nanometer-sized pores both in the plasma membrane and in internal cell membranes, termed "supraelectroporation" [35].

With that said, pore populations are not necessarily uniform, so individual pores can experience stochastic (thermal) size fluctuations and become Pr-permeable. Intense nsEP treatments make Pr uptake detectable [12,13,18,26], although it remains orders of magnitude

Abbreviations: DIC, differential-interference contrast; nsEP, nanosecond electric pulse(s); PMT, photomultiplier tube; Pr, propidium

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weaker than in dead or chemically permeabilized cells. The primary Pr influx through nsEP-opened pores should also be distinguished from a downstream, massive, delayed Pr entry due to membrane rupture in swollen cells [36,37], or due to Ca^{2+}-mediated secondary pore opening or enlargement [38].

Pr entry after intense nsEP treatments could result from increasing either the pore size or the pore number, or both. In the case of the size increase, pores will likely lose the salient features of nanopores, such as voltage and current sensitivity and ion selectivity [14,15]. Alternatively, producing larger numbers of long-lived nanopores without changing their permeability could be an attractive tool both for biophysical studies of nanopore properties and for biotechnological applications.

In this study, we evaluated pore size by comparing the influx of two dye molecules of different size, namely, YP and Pr. We used stained homogenates of lysed cells and confocal imaging to calibrate the fluorescence intensity against dye concentration. We found that YP fluorescence is linearly proportional to dye concentration, whereas Pr fluorescence is linear only down to 4 μM and disproportionally reduced at lower Pr concentrations. This "lag" hampers the detection of low Pr levels and can explain, at least in part, why many studies with nsEP report no detectable Pr entry. After the correction of fluorescence data by the calibrations, we found that (a) uptake of both YP and Pr increases linearly with pulse number, and (b) the molecular ratio of YP and Pr uptake is constant and independent of the pulse number (at least for 5 to 100 pulses). These data are consistent with increasing the long-lived nanopore population without changing the size or permeability of individual pores.

2. Materials and methods

Cell culture methods, nsEP generation and delivery to cells, dosimetry, and image acquisition and processing were essentially the same as reported earlier [6,15,37,39] and will only be described in brief here. A novel method of calibrating the emission of DNA stains against their concentrations is introduced and discussed in Section 3.2.

2.1. Cells and media

CHO-K1 cells (Chinese hamster ovary) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated as described elsewhere [6,37]. For the passage immediately preceding experiments, cells were transferred onto glass coverslips pretreated with poly-L-lysine to improve adhesion. The media and its components were purchased from Mediatech Cellgro (Herdon, VA) except for the serum (Atlanta Biologicals, Norcross, GA).

2.2. Cell imaging

A cover slip with cells was transferred 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). The chamber was filled with a buffer composed of (in mM): 136 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4), with the addition of 5 μg/ml (7.5 μM) of Pr iodide and 1 μM of YP iodide. This ratio of concentrations was established empirically in order to account for the different brightness of the dyes, to enable their reliable detection, and to minimize the chance of quenching (see Section 3.2). The same concentrations are commonly used in membrane permeabilization studies with these dyes. For one experiment illustrated in Fig. 2, the buffer contained 43 μg/ml of Pr and no YP.

The buffer osmolality was at 290–300 mOsm/kg, as measured with a freezing point microosmometer (Advanced Instruments, Inc., Norwood, MA). The chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and Invitrogen (Eugene, OR).

Images were acquired with a 40×, NA 0.95 dry objective. YP and Pr were excited at 488 and 543 nm, and the emission was collected at 505–525 nm and 560–660 nm, respectively. The lasers were operated in a line sequence mode to avoid the "cross-talking" of the dyes.

The sensitivity of emission detectors (photomultiplier tubes, PMT) was chosen individually for different sets of experiments. For those summarized in Fig. 1, the sensitivity was relatively low, to cover a broad range of Pr uptake with possible pixel saturation only in digitonin-permeabilized cells. For Fig. 2, the PMT sensitivity was tuned as indicated in the figure legend. For experiments in Figs. 3–6 and 8, the sensitivity was set constant at the highest level which still prevented pixel saturation after the most intense nsEP treatment (100 pulses). Experiments presented in Fig. 7 were performed separately from the rest of the study and utilized somewhat different laser and PMT settings; calibrations shown if Fig. 3 do not apply to these data.

Stacks of images captured before and after pulsing (in most experiments, at regular 10-s intervals) were quantified with MetaMorph Advanced v.7.7.10.0 (Molecular Devices, Foster City, CA).

2.3. nsEP exposure and dosimetry

The exposure procedures were similar to those described recently [6,15,39,40]. Nearly rectangular 60- or 600-ns pulses were generated in a custom-made transmission line circuit. The electrical energy was stored in an RG 58 (50 Ohm) coaxial cable and released in a pulse upon closing of a fast MOSFET switch (DE275-102N06A). The duration of the electric pulse equaled the round-trip time of the electromagnetic wave in the coaxial cable and therefore was proportional to the length of the cable. The amplitude of nsEP generated in this transmission line and measured across a matched 50 Ohm load was about one half of the charging voltage. The amplitudes and shapes of nsEP were captured and measured with a TDS 3052 oscilloscope (Tektronix, Beaverton, OR). The nsEP rise time (10 to 90%) was 3.4 and 6.2 ns for 60- and 600-ns pulses, respectively. The shape of a 60-ns pulse is illustrated in Fig. 1C (inset).

Pulses were triggered externally by a TTL pulse protocol using Digidata 1322A board and Clampex v. 10.2 software (Molecular Devices, Sunnyvale, CA). The nsEP repetition rate, the number of nsEP, and the synchronization of nsEP exposure with image acquisitions were all programmed in pClamp.

Throughout this paper, the reported time intervals between nsEP and imaging are those between the onset of exposure and the onset of image acquisition. It should be kept in mind that each image acquisition could take up to 5 s, and nsEP exposures could take up to 10 s (depending on the number of pulses).

Pulses were delivered to a selected cell (or a group cells) with a pair of tungsten rod electrodes (100 μm diameter, 100 or 175 μm gap) driven by a robotic manipulator (MP-225, Sutter Instruments, Novato, CA). The electrodes were positioned precisely at 30 or 50 μm above the coverslip surface at a 40° angle to it. Selected cells were in the middle of the gap between the tips of the electrodes. The cells were randomly oriented with respect to each other and the electrodes; we did not notice any consistent impact of cell orientation or size on the effect of nsEP. For sham exposures, all procedures were identical, but no pulses were triggered.

The electric field at the cell location between the electrodes was determined by 3D simulations as described earlier [15], with a finite-element Maxwell equation solver Amaze 3D (Field Precision, Albuquerque, NM). The calculations use a Laplace equation and are based on an electrostatic model (either dielectric or conductive). For bath buffer resistivity on the order of 100 Ω·cm, a dielectric relaxation time would be on the order of 1 ns. Since the nsEP duration was much greater than the relaxation time, we disregarded dispersive properties of the medium [41] and used the conductive model. A closed, grounded boundary using the Dirichlet condition was set 10 times gap distance away from the nsEP-delivering electrodes. The grid size was chosen at