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Quantifying pulsed electric field-induced membrane nanoporation in single cells

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ABSTRACT

Plasma membrane disruption can trigger a host of cellular activities. One commonly observed type of disruption is pore formation. Molecular dynamic (MD) simulations of simplified lipid membrane structures predict that controllably disrupting the membrane via nano-scale poration may be possible with nanosecond pulsed electric fields (nsPEF). Until recently, researchers hoping to verify this hypothesis experimentally have been limited to measuring the relatively slow process of fluorescent markers diffusing across the membrane, which is indirect evidence of nanoporation that could be channel-mediated. Leveraging recent advances in nonlinear optical microscopy, we elucidate the role of pulse parameters in nsPEF-induced membrane permeabilization in live cells. Unlike previous techniques, it is able to directly observe loss of membrane order at the onset of the pulse. We also develop a complementary theoretical model that relates increasing membrane permeabilization to membrane pore density. Due to the significantly improved spatial and temporal resolution possible with our imaging method, we are able to directly compare our experimental and theoretical results. Their agreement provides substantial evidence that nanoporation does occur and that its development is dictated by the electric field distribution.

1. Introduction

Subtle disruptions in the cell membrane can have significant consequences at the whole organism level. For example, several diseases, including cancer and autoimmune disorders, are the result of breakdowns in cell communication or cell signaling [1–10]. In cases like these, the plasma membrane plays a critical role because all extracellular signaling pathways rely on it in some fashion [11–15]. Therefore, an improved understanding of how the cell membrane responds to and interacts with its environment at the molecular level could provide insight into the larger organismal response.

One mechanism the cell utilizes to drive cellular change is to modify either the selective or unrestricted plasma membrane permeabilization [7,16–22]. While there are many possible conditions and methods capable of initiating such a response, artificially increasing permeabilization via pulsed electric fields has proven a useful tool for drug delivery and gene transfection [23–30]. Recent results suggest that the increase in permeability is related to the formation of nanopores within the membrane. These nanopores can form very quickly (~100 ns or less) and can persist for several minutes [31–37].

Using nanosecond pulsed electric fields (nsPEF), it is possible to disrupt the membrane in a controllable manner, enabling methodical investigations into the underlying mechanisms that govern membrane permeabilization. For example, it is believed that the number and location of nanopores created are determined by the duration and the amplitude of the applied nsPEF [32–37]. To date, the majority of research using this approach has either focused on controlled measurements using artificial lipid bilayer structures, such as micelles or vesicles, or has employed indirect methods of nanoporation detection, such as diffusion of fluorescent probes across the membrane disruption. Both techniques provide useful insights but are unable to directly detect and completely capture the dynamic nature of the poration and recovery process in live cells at the necessary spatial and temporal rates [33,34,36,38,39].

Recently, we developed an optical technique that overcomes these challenges, making rapid structural measurements of the membrane possible [31]. This live-cell imaging method combines a lipophilic probe molecule, Di-4-ANEPPDHQ (Di-4) and the selection criteria of second harmonic generation (SHG) to monitor rapid disruption of the plasma membrane. Therefore, this technique directly probes nanoporation, providing a quantitative measurement. This is a departure from the traditional use of a dye's fluorescence properties to report on transmembrane potential [39,40]. Because fluorescence has no

Abbreviations: nsPEF, nanosecond pulsed electric field; Di-4, Di-4-ANEPPDHQ; SHG, second harmonic generation; SE, Smoluchowski equation; RC, resistor-capacitor; SNR, signal-to-noise; Jurkat, Jurkat clone E6-1 human T-lymphocytes.

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symmetry requirements, it is limited to an inference of nanoporation based on the hypothesis that increased transmembrane voltage (generally in excess of ~1 V) is the key to pore development and that dye crosses the membrane preferentially through these aqueous pores [35,38–40]. It is also distinct from methods correlating the electro-optic response of the dye with transmembrane voltage through SHG [41].

SHG is a nonlinear optical process where an incident electromagnetic field of sufficient intensity is capable of inducing oscillating dipole moments among an ensemble of scattering molecules. The contribution of the resulting dielectric polarizations of each molecule can be summed to describe a radiated electromagnetic field that oscillates at twice the frequency of the incident beam. The phenomenon is predicated on both the molecule's environment and its orientation. SHG is only permitted if the molecule is in a noncentrosymmetric environment, like an interfacial layer, and the phase matching condition is satisfied [31,42,43].

In our experiment, these conditions can only be met when probes are embedded in an intact membrane (Fig. 1). The lipophilic nature of the SHG probes allows the outer leaflet of the cell's lipid bilayer to act as a scaffolding nanostructure, enabling self-assembly and alignment [44]. However, if the membrane becomes porated, the probe's environment is no longer noncentrosymmetric, and the requirements for appreciable SHG are violated. In this disordered state, the probes are no longer optimally aligned and the signal from that area of the plasma membrane will decrease. Therefore, by analyzing the intensity of the SHG signal, it is possible to determine if the environment immediately around the probe is ordered or disordered.

Consequently, the differential SHG signal at any location in the cell membrane before and after nsPEF exposure directly correlates with the nanopore density at that location. It is also worth noting here that the probes themselves are not damaged during this process, nor do they diffuse out of the membrane. Therefore, using this method, the poration and recovery of the membrane can be studied multiple times on the same cell. In a previous study, we employed such an approach to demonstrate recovery of SHG signal on time scales similar to that of the pore lifetime (several minutes) [31].

In the present work, we use SHG imaging to detect changes in the membrane order in response to nsPEF. Given the brevity of the pulse compared to Di-4′s reported response time (on the order of 10 s of ms), we can neglect any electrical interaction with the probe and allow the local noncentrosymmetry and selection criteria of SHG to regulate the signal [45,46]. We measure the behavior as a function of both pulse duration and amplitude. Additionally, we adapt a circuit-based model of the membrane to explain the cell's response to nsPEF. By correlating the predicted pore density with the loss of SHG signal, we provide a more accurate and quantitative picture of electrically-induced membrane disruption.

2. Materials and methods

2.1. Planar membrane patch model

Circuit-based models have been used extensively throughout the bioelectrics research community to develop an intuition about the dynamic response of the membrane to electric fields [36,47,48]. In this approach, each component of the cell is correlated with an equivalent electrical counterpart. This method allows researchers to construct

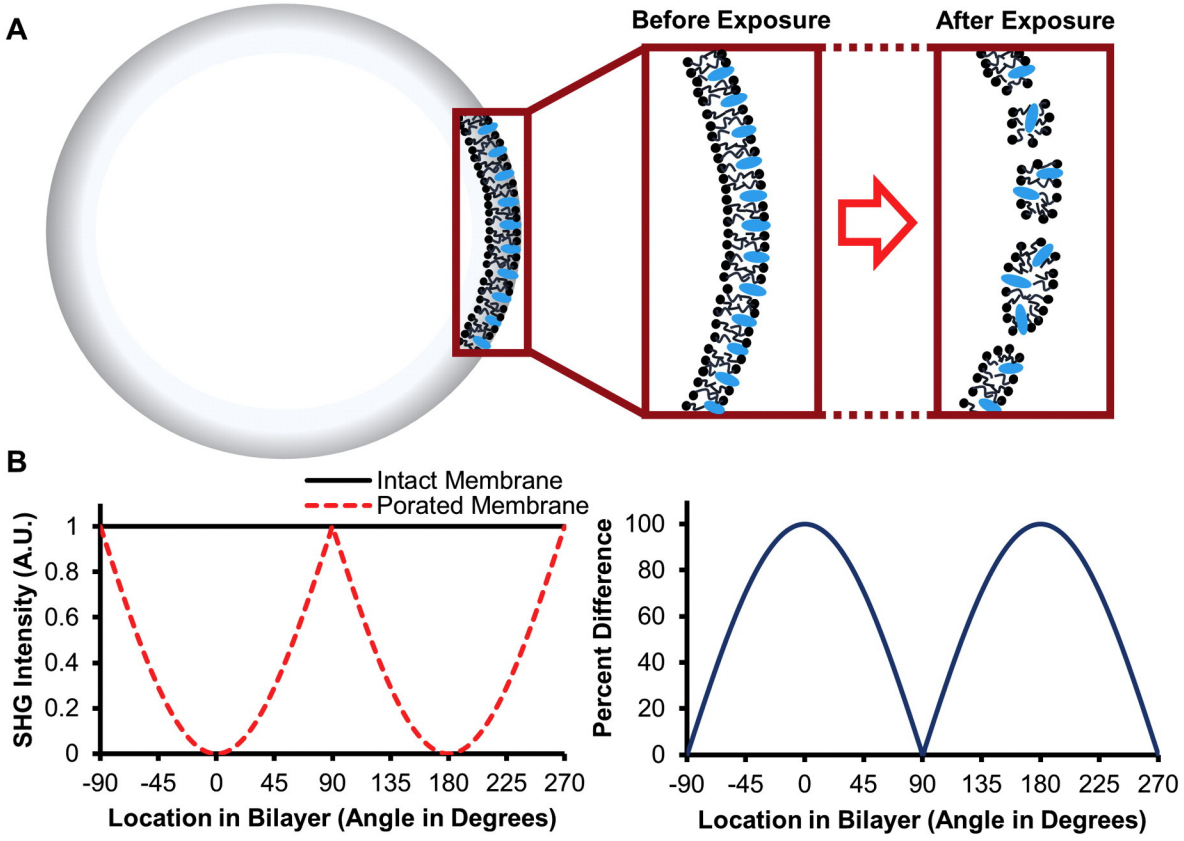

Fig. 1. Using SHG dyes to detect membrane nanoporation. A) The plasma membrane of the cell acts as a template for the SHG dyes, orienting them with respect to the incident field and maximizing SHG emission signal (Right and Center). When the cell membrane is disrupted by nsPEF exposure (Left), the dyes lose this orientation, and the signal is reduced. B) Because emission is directly correlated to the membrane order, the membrane organization at a specific location can be determined. Any change between the two states, intact and porated, can be quantified via a percent difference in SHG intensity.