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Effects of extracellular calcium on cell membrane resealing in sonoporation

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Abstract

Sonoporation has been exploited as a promising strategy for intracellular drug and gene delivery. The technique uses ultrasound to generate pores on the cell membrane to allow entry of extracellular agents into the cell. Resealing of these non-specific pores is a key factor determining both the uptake and post-ultrasound cell survival. This study examined the effects of extracellular Ca^{2+} on membrane resealing in sonoporation, using *Xenopus* oocytes as a model system. The cells were exposed to tone burst ultrasound (1.06 MHz, duration 0.2 s, acoustic pressure 0.3 MPa) in the presence of 0.1% Definity® at various extracellular [Ca^{2+}] (0–3 mM). Sonoporation inception and resealing in a single cell were monitored in real time via the transmembrane current of the cell under voltage clamp. The time-resolved measurements of transmembrane current revealed the involvement of two or more Ca^{2+} related processes with different rate constants and characteristics. Rapid resealing occurred immediately after ultrasound application followed by a much slower resealing process. Complete resealing required [Ca^{2+}] above 0.54 mM. The cells resealed in 6–26 s at 1.8 mM Ca^{2+}, but took longer at lower concentrations, up to 58–170 s at 0.54 mM Ca^{2+}.

Keywords: Sonoporation; Ultrasound; Membrane pore resealing; Calcium; Voltage clamp

1. Introduction

Safe and efficient intracellular delivery of drugs and genes is critically important in targeted cancer treatment and gene therapy applications. Many studies have demonstrated that ultrasound (US) can deliver DNA, proteins, and other formulations of molecules of interest into viable cells [1–8]. It has been hypothesized that US energy, often amplified by micro-bubble activities (e.g. bubble oscillation and collapse) [9–16], generates transient, non-specific pores on the cell membrane, a process termed sonoporation [14,17–19]. The transient pores allow a limited time window for the otherwise non-permeable extracellular agents to enter the cell.

Because non-ionizing US exposure can be non-invasively controlled in application location and duration, sonoporation may provide an advantageous, safe delivery strategy for *in vivo* applications. Compelling results obtained recently have stimulated great interest in the development of sonoporation applications with exciting possibilities[1–8]. However, progress in the field is hindered by a lack of mechanistic understanding of the sonoporation process and its outcome beyond demonstrations of initial feasibility [20]. Challenges in several key aspects remain to be addressed before sonoporation can be used successfully in humans as an efficient and safe strategy: 1) lack of means to rationally determine optimal sonoporation parameters, including physical and biochemical factors, to ensure high delivery efficiency and consistent outcome; 2) lack of mechanistic understanding of the causes for the downstream, cellular bio-effects and organ-level impacts of sonoporation; 3) lack of valid correlation and capability for translating in situ results to in vivo environment. Clearly, tackling these difficult yet important tasks requires in-depth investigation of the sonoporation process and identification of the major factors affecting it.

The observation of stably enhanced intracellular uptake of markers and the expression of intentionally delivered genes in viable cells via sonoporation indicate the transient nature and small scale of the membrane poration process. The US

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generated pores on the plasma membrane must reseal to prevent the loss of intracellular contents to ensure cell survival, thereby limiting efficient inward transmembrane passage of desired extracellular agents within a time window before the completion of pore resealing. Furthermore, repair of the membrane disruption is necessary to avoid intracellular overload of ions that might be toxic to the cell or serve as the triggering sources for other irreversible and reversible cellular processes such as apoptosis [21] and calcium oscillation [22], making the rate of resealing one of the key factors determining the uptake efficiency and post-ultrasound cell fate.

It is therefore of significance to understand the process of sonoporation resealing, yet it is a task challenged by the lack of appropriate techniques to study the transient and sub-micron process. Consequently, sonoporation studies have been largely limited to static post-US assays. While important knowledge can be obtained through such analysis, post-US assays inevitably overlook the actual transient process of cell poration. Selection and attempted optimization of sonoporation parameters have mainly relied on empirical results of delivery outcome obtained after sonoporation. However, it is likely that the US parameters determined this way are only associated with specific experimental conditions due to the complexity of the US–cell interaction and its coupling with surrounding bubble activities. Given the statistical variance of such interactions in large number of cells, post-US assays are usually inadequate to deterministically correlate ultrasound parameters with sonoporation outcome and to uncover cellular mechanisms of sonoporation in individual cells.

To address these challenges and to understand the mechanism of sonoporation, we demonstrated previously for the first time the feasibility of studying sonoporation at the single cell level in real time using the voltage clamp techniques [18,23]. The transmembrane current of single cell under voltage clamp was measured to assess the change of cell porosity in sonoporation. Before US application, the transmembrane current is close to zero at a constant membrane holding potential (voltage clamped) in the absence of activation of endogenous ion channels, as the whole cell membrane is regarded as a resistor with constant resistance [24,25]. In sonoporation, US generates pores on the membrane (the pores effectively reduce the membrane resistance), which allows ions to flow through the pores and results in change in transmembrane current. The transmembrane current is determined by the pore size and ion concentration gradient across the cell membrane. Therefore the transmembrane current can be used as a sensitive means to monitor the dynamics of US induced pores in a single cell with high temporal resolution and sensitivity [18]. The novel application of such electrophysiological techniques enables a time-resolved measurement of sonoporation at single cell level, providing a sensitive and quantitative means to investigate the sonoporation process. Our previous results demonstrated that calcium in the extracellular solution affects sonoporation resealing [18]. The current study focused on quantitative investigation of the effects of extracellular Ca^{2+} on the resealing of US induced membrane disruption; results of the study might provide important molecular insight into reversible sonoporation to guide optimal delivery outcome.

2. Methods

2.1. Xenopus oocytes preparation

Commonly used as a model system for electrophysiological recordings, the *Xenopus* oocyte was chosen as a membrane model system [18,23,26,27] to study sonoporation dynamics. The same protocol described in our previous papers [18,23] was used for this study, and the procedures of harvesting and preparation of *Xenopus* oocytes follow an animal protocol approved by our Institutional Animal Care and Use Committee. Briefly, adult *Xenopus laevis* females (NASCO, Fort Atkinson, WI) were anaesthetized by immersion in a 0.3% tricaine (Sigma, St. Louis, MO) solution for 20 min. Oocytes were taken through a small incision (0.5–1 cm) made in the frog's lower abdomen, which was subsequently sutured back together. The frogs were allowed to recover in fresh water. Oocytes were digested in collagenase (1 mg/ml in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, with pH 7.60), followed by a manual defolliculation if necessary. The oocytes were used immediately in experiments or stored in ND96 solution at 18 °C for one or two days before use. The oocytes were placed into the ND96-based test solution with the desired [Ca^{2+}] and [Mg^{2+}] several (~3 to 7) min before sonoporation experiment. Each oocyte was used for only one exposure of US in the sonoporation experiment. The cells were kept in culture for up to 24–36 h after sonoporation to determine the survival rate.

2.2. Experimental setup for real time measurement of sonoporation

A 35 mm polystyrene BD Falcon™ bacteriological Petri dish (Fisher Scientific, Pittsburgh, PA) was used to house a single *Xenopus* oocyte (diameter 0.8–1.1 mm) containing 4 ml ND96 or modified ND96 (altered [Ca^{2+}] or [Mg^{2+}]) solution with or without 0.1% Definity™ (Bristol-Myers Squibb Medical Imaging, N. Billerica, MA). The Petri dish has been tested to show to have minimal effect (>95% transmission) on the transmission of US pulses through the bottom. Definity™ is an US imaging contrast agent consisting of stabilized bubbles with a diameter of 1.1–3.3 μm (mean 2.2 μm) and was used as cavitation nuclei to facilitate sonoporation. The solution with Definity™ was made by diluting the original stock solution of Definity™ (~1.2×10^{10} particles/ml) by 1000 times in ND96 solution. This concentration is within the range of in vivo concentration at meaningful locations resulted from a typical imaging dosage (10^5–10^7 particles/ml). The actual bubble concentration in the experiments was measured by counting the bubbles in selected sample volumes under the microscope before and after exposures to US.

Tone-burst US pulses of the desired duration and pressure amplitude were generated using an unfocused circular planar piezoelectric US transducer (Piezo Technologies, Indianapolis, IN) with a diameter of 2.54 cm and a center frequency 1.075 MHz, driven by a function/waveform generator (Model 33250A, Agilent Technologies, Palo Alto, CA) and a 75-W power amplifier (Model 75A250, Amplifier Research, Souderton, PA).