Enhanced Electroporation in Plant Tissues via Low Frequency Pulsed Electric Fields: Influence of Cytoplasmic Streaming

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Pulsed electric fields (PEF) are known to be effective at permeabilizing plant tissues. Prior research has demonstrated that lower pulse frequencies induce higher rates of permeabilization, but the underlying reason for this response is unclear. Intriguingly, recent microscopic observations with onion tissues have also revealed a correlation between PEF frequency and the subsequent speed of intracellular convective motion, i.e., cytoplasmic streaming. In this paper, we investigate the effect of cytoplasmic streaming on the efficacy of plant tissue permeabilization via PEF. Onion tissue samples were treated with Cytochalasin B, a known inhibitor of cytoplasmic streaming, and changes in cellular integrity and viability were measured over a wide range of frequencies and field strengths. We find that at low frequencies ($f < 1$ Hz), the absence of cytoplasmic streaming results in a 19% decrease in the conductivity disintegration index compared with control samples. Qualitatively, similar results were observed using a microscopic cell viability assay. The results suggest that at low frequencies convection plays a statistically significant role in distributing more conductive fluid throughout the tissue, making subsequent pulses more efficacious. The key practical implication is that PEF pretreatment at low frequency can increase the rate of tissue permeabilization in dehydration or extraction processes, and that the treatment will be most effective when cytoplasmic streaming is most active, i.e., with freshly prepared plant tissues. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.*, 28: 445–453, 2012
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Introduction

Pulsed electric field (PEF) treatment is an advanced food processing technology for the dehydration and extraction of biological materials.[1–7] PEF shows great potential because of its ability to preserve the nutritional value of fresh produce[8–10] while minimally affecting sensory properties.[4] During a typical PEF treatment, a DC voltage is applied periodically as a number of short pulses, each with duration of a few microseconds. With sufficiently high electric field strengths or number of pulses, PEF treatment causes membrane breakdown within the cells (i.e., electroporation), and consequently expedites the rate of water removal and increases the yield of juice extraction from within the tissue. PEF treatment has been studied in a wide variety of plant tissues, including potato, carrot, alfalfa, apple, sugar beet, and onion; in each case, it has been shown that increasing the field strength or number of pulses increases the efficacy of permeabilization.[1,10–13] Fewer studies, however, have examined the effect of pulse frequency. Lebovka et al. reported that a pulse frequency of 0.2 Hz caused significantly more permeabilization in apple tissues than 100 Hz.[11]

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Asavasanti et al. explored the effect of frequency in more detail with onion tissues and showed that there was a critical frequency of ~1 Hz below which permeabilization was significantly higher.[7] Moreover, Asavasanti et al. reported a correlation between the PEF frequency and the subsequent speed of intracellular convective motion, i.e., cytoplasmic streaming. The main purpose of this paper is to examine the role cytoplasmic streaming plays in the mechanism of PEF permeabilization at low frequencies.

Reportedly discovered by Bonaventura Corti in 1774,[14–16] cytoplasmic streaming is driven by molecular motor proteins (including for example kinesin and myosin) that utilize energy released from ATP conversion to pull subcellular particles along bundled actin microfilaments, thereby inducing fluid motion both in the cytoplasm and the vacuolar fluid.[14,16–18] In plant cells, observed flow patterns include unidirectional streaming, fountain streaming, and spiral rotational streaming. In 1885 De Vries theorized that this convective fluid motion is essential for metabolism and distribution of nutrients, enzymes, and larger particles, such as plastids and organelles, within living cells[14–16,18,19]; more recent work has corroborated this view of the biological role of cytoplasmic streaming.[15,16,18] In addition, streaming may also enhance the exchange of materials between organelles, as well as between adjacent cells.[14,16-18]

Early studies often associated the existence of cytoplasmic streaming with cell viability because loss of membrane permeability results in cessation of cytoplasmic streaming.[20–22] Several researchers reported that application of electrical stimuli results in cessation or sudden decrease in the observed speed of cytoplasmic streaming, but little is known about the underlying mechanism.[21,23–25] Several hypotheses have been proposed, including generation of membrane electrical responses (or action potentials)[24,26,27], change in the chemical composition or the physical structure of the cytoplasm[14,28], and temporary disappearance of the driving force for the streaming.[14,25] Although the start of the action potential is always followed by the cessation of cytoplasmic streaming, cytoplasmic streaming can also be halted without triggering any action potential.[25,26] The temporary disappearance of the motive force, i.e., an interruption in the conversion of ATP to mechanical energy, appears to be the most widely accepted mechanism behind cessation of cytoplasmic streaming.[25,29,30] There is a strong correlation between ATP concentration and the observed speed of cytoplasmic streaming, while many chemicals including ADP, orthophosphate, pyrophosphate, and sulfate are known to inhibit cytoplasmic streaming, apparently by competition with ATP binding sites.[15,29,31] A sufficiently strong electrical stimulus causes membranes to lose their ability to regulate intracellular ionic concentrations, resulting in transient increases in Ca^{2+} concentration in the cytoplasm. At sufficiently high concentrations, Ca^{2+} is cytotoxic and can deactivate the molecular motors driving the cytoplasmic streaming.[16,21,25,26,32–34] Cytoplasmic streaming is known to restart when the normal ionic balance in the cytoplasm is restored by Ca^{2+} pumps.[32,33]

Most previous studies on the effect of electrical stimuli on cytoplasmic streaming were performed using direct current (DC) or alternating current (AC) electric fields.[21,23–25,28] To our knowledge, our recent work was the first report of a correlation between the frequency of pulsed electric fields (PEF) and observed cessation of cytoplasmic streaming.[7] Specifically, in onion tissues streaming stopped abruptly at high pulse frequency, but the streaming gradually slowed down at pulse frequencies lower than 1 Hz. Notably, PEF is known to be more effective at low frequencies.[35–38] We hypothesize here that the persistence of cytoplasmic streaming during low frequency PEF plays a role in accelerating physical damage of the cell membranes. We test this hypothesis directly by assessing the efficacy of PEF in onion tissues where cytoplasmic streaming is chemically deactivated. A cytoplasmic streaming inhibitor (Cytochalasin B) was applied to onion tissues, and the degree of tissue damage and cell integrity loss were investigated in onion epidermis treated with PEF over a range of pulse frequencies. Cell viability after PEF was also evaluated using the stain neutral red (NR) and a viability staining technique.

Materials and Methods

Sample preparation

Spanish yellow onions (*Allium cepa*) were obtained from Gills Onions (Oxnard, CA) and kept in cold storage at 4°C up to three months. Onion bulbs 7.5–9 cm in diameter were selected to standardize maturity. Prior to sampling, onion bulbs were equilibrated at room temperature (~25°C) for an hour before removing the papery outer scales and the first fleshy scale. The epidermis was manually stripped from the top (inner) surface of the second outermost fleshy scale of the onion bulb. Onion flesh debris underneath the epidermis was removed by rinsing with deionized water and gentle rubbing for 60 seconds. Onion epidermis was placed on a glass slide and cut into 5 mm × 5 mm sections using a sharp razor blade. Samples were stored in 0.2 M mannitol solution (isotonic solution) until use, but for no longer than 3 hours. The isotonic concentration was determined by bathing onion epidermis in mannitol solutions of different concentrations and observing changes in epidermal cell size. At the isotonic concentration, no change in epidermal cell size was observed during 3 hours of storage. The isotonic concentration was determined for each lot of samples in three replicates.

Pulsed electric field (PEF) treatment

The PEF treatment chamber was similar to that used previously by Asavasanti et al.[7] Briefly, the onion epidermis was placed between two parallel copper electrodes attached to a glass slide (Figures 1a,b); the distance between the two electrodes for all experiments was 3 mm. The onion section was oriented such that the long axes of the cells were parallel to the electrodes and the waxy side (epidermis) facing up. Approximately 30 μL of isotonic solution was used as a mounting medium, and a cover slip was carefully placed on top of the chamber so as to avoid entrapment of air bubbles. The cover slip was thus separated from the tissue by a thin layer of liquid, held in place by capillary forces. Pulsed electric fields were applied using a specially designed system to deliver monopolar positive pulses of rectangular shape (Figure 1c). The pulse width t_p was fixed at 100 μs, which is typical and representative of pulse widths employed previously for a variety of plant tissues.[3,5,10] The PEF system consisted of a high voltage power supply (PowerPAC HV, BIO-RED, Hercules, CA), a function generator (model 33220A, Agilent, Santa Clara, CA,), a PEF generator, a sample holder, and an oscilloscope (model TDS1012B, Tektronix, Beaverton, OR) for signal monitoring. The electric field strength (0–500 V/cm), number of pulses (0–150 pulses),

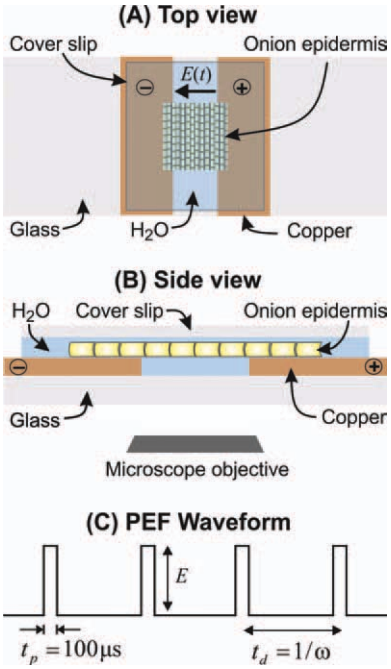

Figure 1. Schematic diagrams of the PEF apparatus and waveform.

(a) top and (b) side views of the PEF apparatus (not to scale). The copper electrodes are connected to the PEF generator unit during application of the PEF waveform and then to the LCR meter for subsequent conductivity characterizations. A typical PEF waveform is shown in (c).

and pulse frequency (0.1–1,000 Hz) were all varied systematically to determine the effect on the viability and integrity of the onion epidermis. The control samples were placed on the treatment chamber for 1 min without applying any electric field and then analyzed identically to the PEF-treated samples.

Inhibition of cytoplasmic streaming

To study the effect of cytoplasmic streaming on PEF efficacy, some of the onion epidermis samples were chemically treated with Cytochalasin B (CB, C6726-1MG, Sigma-Aldrich, St. Louis, MO), a known inhibitor of cytoplasmic streaming[29,39–43], prior to observation. The drug is sparingly soluble in water, so stock solutions were prepared in 1% aqueous dimethylsulphoxide (DMSO, D128-500, Sigma-Aldrich, St. Louis, MO). The final concentration of 30 μg/mL (6.3×10^{-5} M) was prepared by diluting stock solution with isotonic solution at 1% (*v*/*v*). To ensure that DMSO by itself did not have a deleterious effect on the onion samples, separate control experiments were performed with 1% DMSO solutions without any added Cytochalasin B. Prior to each experiment, each onion sample was bathed with 30 μL of either Cytochalasin B solution, 1% DMSO solution, or isotonic solution. The resulting cytoplasmic streaming velocities were recorded with video microscopy to determine the effect of the different solutions on both the streaming speed and the time required for cessation of cytoplasmic streaming.

Characterization of cytoplasmic streaming

The samples were observed with a phase contrast, inverted light microscope at an objective magnification of 20× (Leica DMI3000B, JH Technologies, San Jose, CA). The video was captured from the mid section of the sample with a CMOS monochrome USB camera (EO-3112M, Edmund Optics Inc., Barrington, NJ) at a frame rate of 12 frames per second and a resolution of 1,280 × 1,024 pixels. The initial cytoplasmic streaming profile was recorded for each sample prior to bathing in isotonic (control), 1% DMSO, or Cytochalasin B solutions. The video was captured at different time intervals, i.e., more frequent in the beginning and every 10 min later on, over the period of 1 hour.

Quantitative information about cytoplasmic streaming speed was extracted from the videos using custom image analysis routines in MATLAB version 7.2.0.232 (Mathworks Inc., El Segundo, CA). The speed of cytoplasmic streaming at any particular point in time was estimated by measuring the distance that an individual organelle moved between frames in the movie (i.e., over a time period of 83 ms). For each treatment, an average speed was estimated from two separate onion samples, where 24 total locations of about 60 × 60 pixels were analyzed. Three areas were selected to represent the cytoplasmic streaming speed profile within each cell, and a total of four cells were analyzed.

Determination of cell integrity: electrical conductivity disintegration index

In biological tissues, pores formed in the cell membranes upon application of the electric field result in an increase in electrical conductance since ions may easily pass through the electroporated membrane; accordingly, conductivity serves as a measure of the loss of plant tissue integrity. The electrical conductance G of onion epidermis before and after PEF treatment was measured with an LCR meter (model 4284A, Hewlett-Packard, U.S.A.) at a frequency of 20 Hz. The applied potential across the sample was set at 1 V. At least 10 samples each were used to determine the electrical conductance of onion epidermis treated with PEF and the controls (no PEF). The degree of tissue damage was quantified using the electrical conductivity disintegration index Z^*,[44]

$$Z^* = \frac{\sigma - \sigma_i}{\sigma_d - \sigma_i} = \frac{(G - G_i) * L/A}{(G_d - G_i) * L/A} = \frac{G - G_i}{G_d - G_i} \qquad (1)$$

where σ is the measured electrical conductivity (S/cm), L is the sample thickness (m), A is cross-sectional area of the sample (m^2), L/A is the cell constant (cm^{-1}), and the subscripts "i" and "d" refer to the conductivities of intact and completely ruptured tissue, respectively. From Eq. 1, $Z^* = 0$ for intact tissue (control, no PEF) and $Z^* = 1$ for completely ruptured tissue.

Determination of cell viability: viability staining and light microscopy

Control and PEF-treated onion epidermal layers were also examined using viability staining and microscopic analysis. Neutral red (NR) stain (Standard Fluka, Sigma-Aldrich, St. Louis, MO) solution was freshly prepared each time before staining the samples to avoid precipitation and pH change. NR dye solution was prepared by diluting 2% NR dye in a dilution solution for 15 min, then filtering twice using Whatman filter paper no. 1 (Whatman International Ltd., Springfield Mill, Maidstone, Kent, UK). The dilution solution was prepared from deionized water with 0.2 M mannitol and 0.01 M HEPES (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethane-

sulfonic acid]) (Sigma-Aldrich, St. Louis, MO) buffer. The dilution solution was adjusted to pH 7.8 by addition of 0.1 M NaOH (Sigma-Aldrich, St. Louis, MO).

Approximately 5 μL of NR stain solution was applied to the cuticle-free surface (interior face) of an onion epidermis and allowed to remain in contact for 1 min. The stain solution was removed by gentle blotting with a tissue (Kimwipes, Kimberly-Clark Global Sales, Inc.) and rinsing twice for 1 min each time with 10 μL of isotonic solution.

To obtain images of stained cells, an onion epidermis was mounted on a microscope slide with a drop of dilution solution, a cover glass was applied, and immediately observed with a light microscope (Leica DMIL LED, Leica Microsystems Inc., Bannockburn, U.S.A.) at 4.0× objective magnification. A CMOS color USB camera (EO-3112C, Edmund Optics Inc., Barrington, NJ, U.S.A.) attached to the microscope was used to capture images. Color photomicrographs (2,048 × 1,536 pixels resolution, white balance corrected) were captured from the mid-section of an onion epidermis. Light intensity was set at 50% of the maximum level and an exposure time of 22 ms was selected.

Control samples were stained 1 min after they were placed in the treatment chamber without PEF, whereas PEF-treated samples were stained about 1 min after the treatment. At least three replicates per treatment were conducted on two separate days to allow qualitative and quantitative comparisons between micrographs obtained from the different treatment conditions. A cell count method was used to quantitatively determine the percentage of stained cells, which reflects the overall tissue viability.[45]

Statistical analysis

Data were analyzed using SAS 9.2 software (SAS Institute Inc., Cary, North Carolina, U.S.A.) to determine interaction and individual effects. The effect of each individual PEF parameter on the integrity of onion epidermis was determined whereas the other parameters were fixed. One way ANOVA and Duncan's multiple range tests were used to determine statistically significant differences among different treatment levels ($P < 0.05$).

Results and Discussion

Determination of PEF parameters

Since the goal of this work is to elucidate the effect of cytoplasmic streaming on tissue response to PEF, a crucial first step was to identify PEF parameters that would allow the influence of streaming to be measured; if the field strength was too high (or too weak), either all (or none) of the cells would be permeabilized and no effect would be measured. Accordingly, preliminary experiments were performed to identify optimal values for the electric field strength and pulse number.

The experiments confirmed that our samples of onion epidermis responded to PEF treatment in a fashion similar to previous observations (Figure 2). For single pulse treatments ($n = 1$), there was no significant difference in the conductivity disintegration index, Z^*, between the controls and samples treated at low field strengths, $E = 150$ or 333 V/cm ($P < 0.05$). The electrical field strength threshold for an observable effect of the single pulse treatment was between 333–500 V/cm, which is in qualitative agreement with the

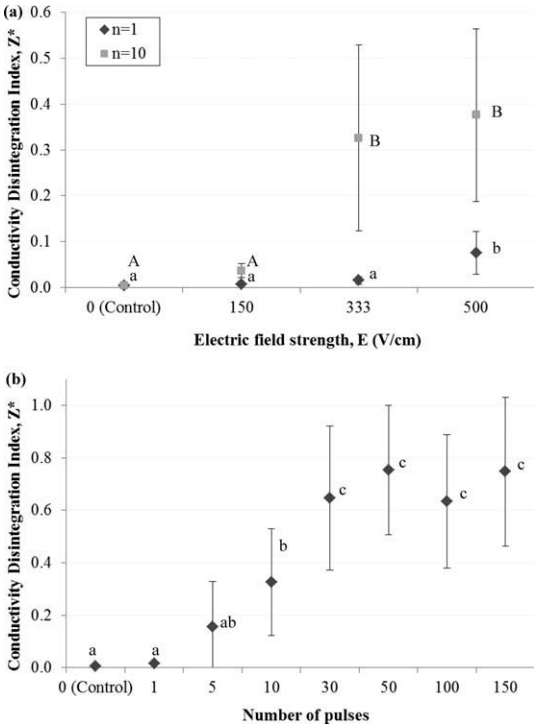

Figure 2. Effect of different PEF parameters on the conductivity disintegration index of onion epidermis.

(a) Effect of electrical field strength on onion epidermis treated at $t_i = 100$ μs, $f = 1$ Hz, and $n = 1$ or 10 pulses (at least eight replicates for all treatments). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$). Statistical comparisons were done separately for single pulse and 10 pulse treatments, and are indicated as lower or upper case letters, respectively. (b) Effect of number of pulses on onion epidermis treated at $E = 333$ V/cm, $t_i = 100$ μs, $f = 1$ Hz (at least five replicates for all treatments). Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

threshold level of 133 V/cm for white onion scale tissue reported by Asavasanti et al.[10] and 350 V/cm for red onion epidermis reported by Fincan and Dejmek.[46] In contrast, for $n = 10$ pulses applied at a frequency of 1 Hz, an electrical fieldstrength of 333 V/cm caused a significant increase in Z^* ($P < 0.05$) from $Z^* = 0.04$ to $Z^* = 0.33$. The effect of pulse number at $E = 333$ V/cm is shown in Figure 2b. The conductivity disintegration index increases gradually between $n = 1$ and $n = 50$, but little increase is seen for $n > 50$. Again, the effect of pulse number is qualitatively consistent with our previous observations.[10]

The data in Figure 2 make clear that appropriate intermediate choices for the electric field strength and number of pulses are $E = 333$ V/cm and $n = 10$, which yielded a conductivity disintegration of ~0.3 (i.e., ~30% of the cells were permeabilized). Accordingly, all subsequent measurements of the effect of cytoplasmic streaming on the response to PEF were conducted using these values.

Inhibition of cytoplasmic streaming by cytochalasin B

We used Cytochalasin B (CB), a cell membrane-permeable mycotoxin, as a cytoplasmic streaming inhibitor. CB inhibits cytoplasmic division by blocking the formation of contractile microfilaments, and consequently has been widely used to inhibit cytoplasmic streaming and cell motility in many plant systems including *Nitella* internode cells and *Avena*

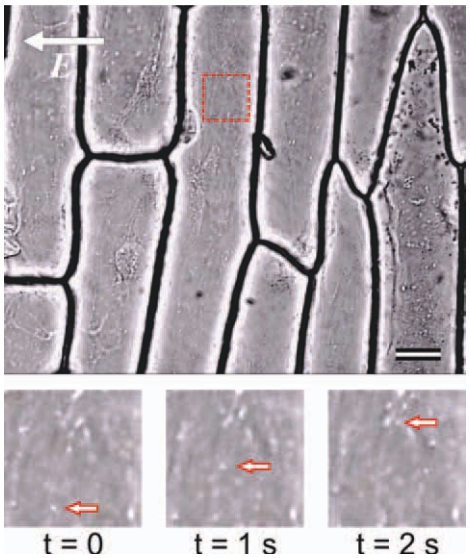

Figure 3. Cytoplasmic streaming in onion epidermal cells.

Top: Representative micrograph of onion epidermal cells viewed with a phase contrast microscope. Scale bar is 50 μm. Bottom: Time lapse images of the region indicated by the dashed square in the top image. Arrows highlight the motion of an individual organelle. Each time lapse image is 50 by 50 μm.

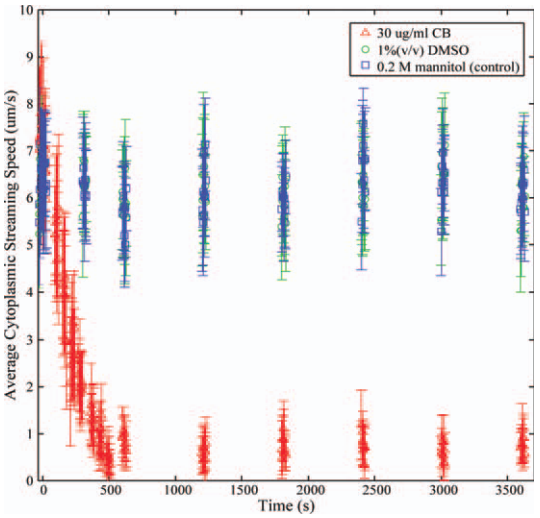

Figure 4. Comparison of the effects of DMSO and CB on cytoplasmic streaming in onion epidermis as a function of time, extracted from video microscopy measurements illustrated in Figure 3.

The average streaming speed at each time interval was evaluated from at least three separate locations in four representative cells from two different samples. At $t = 0$ s, 30 μL of 1% DMSO or 30 μg/mL of CB was applied to the cells.

coleoptile parenchyma cells[41,43] as well as in cultured mammalian cells.[39,40] Sensitivity to the drug varies for different cell types and species. A working concentration of 30 μg/mL was selected based on previous work with *Avena* coleoptile parenchyma cells, in which 30 μg/mL of CB caused cytoplasmic streaming to cease in 30–60 mins.[40,41]

Figure 3 shows a representative observation of cytoplasmic streaming in onion epidermal cells as acquired via video microscopy. The bright objects are organelles which initially travel along microfilaments at several microns per second. Observed flow directions included unidirectional streaming and fountain streaming (in which the motion near the central axes of the cell is opposite to that near the periphery). We emphasize that the magnitude and direction of cytoplasmic streaming vary within each cell, so the speed was measured in three locations throughout the cell; the reported speeds thus represent spatially averaged quantities.

The effect of CB on the speed of cytoplasmic streaming in onion epidermal cells is shown qualitatively in Figure 4. The streaming speed was calculated as the observed particle displacement per unit time interval for all plastids moving through a given location, using the same procedure as described in our previous study.[7] The observed cytoplasmic streaming speed in the control samples (i.e., onion epidermis in isotonic solution) was approximately 6 ± 2 μm/s, which is broadly consistent with previous observations of cytoplasmic streaming speeds ranging from 1–100 μm/s, with a typical speed of 10 μm/s.[7,14] Notably, the control sample showed little variation in speed over a one-hour period. At much longer times, however, the speed gradually declined; it took ~6 hours for the control streaming speed to decrease to 50% of its initial speed (data not shown). The gradual reduction of cytoplasmic streaming over time is presumably because of depletion of ATP to drive this active transport.

Because 1% DMSO was used to prepare the CB solutions, a second experiment to gauge the effect of DMSO (without any CB) on cytoplasmic streaming was also performed. No significant difference in speed was observed between the controls and the 1% DMSO samples (Figure 4). We conclude that 1% DMSO by itself has no effect on the speed of cytoplasmic streaming, in agreement with previous work in which DMSO has been observed and reported to have no effect on cytoplasmic streaming in *Avena* coleoptile parenchyma and *Nitella* internode cells over 50 hours of observation.[41]

In contrast, the tissues treated with 30 μg/mL of CB exhibited a rapid decrease in the speed of cytoplasmic streaming. The streaming speed dropped to about 1 ± 0.5 μm/s, which is consistent with random motion induced by the thermal forces (i.e., Brownian motion), within 5 min of CB application. In addition, the speed remained ~1 μm/s throughout the observation period. The 5-min time period required for 30 μg/mL CB to inhibit cytoplasmic streaming in onion epidermal cells is about a factor of six lesser than the 30–60 min required for *Nitella* internode cells or *Avena* parenchymal cells reported in previous studies.[40,41] This result suggests that onion epidermal cells are more sensitive to CB than these other experimental systems. To ensure that cytoplasmic streaming inside each cell is inhibited, all subsequent experiments reported here used a 30 min incubation time after application of CB to the epidermal tissue.

Effect of cytoplasmic streaming on cell integrity: conductivity disintegration index (Z)*

Our previous study showed that PEF treatment reduced or halted cytoplasmic streaming, with the rate at which the speed decreased sensitive to the PEF frequency, i.e., the higher the frequency the more rapid the cessation of cytoplasmic streaming.[7] We emphasize that our microscopic observations of the intracellular motion provided no indication of electrically generated fluid flow (e.g., electrophoresis or electroosmosis), presumably because each PEF pulse was so short-lived. Instead, we observed a gradual decay in the

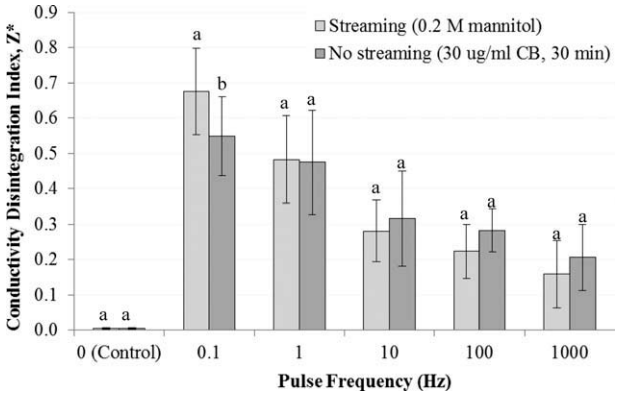

Figure 5. Effect of cytoplasmic streaming on the conductivity disintegration index of onion epidermis treated with PEF at $E = 333$ V/cm, $t_i = 100$ μs, $n = 10$ pulses (at least 10 replicates for all treatments).

Statistical comparisons were done separately for each frequency of PEF treatment, i.e., $f = 0.1$, 1, 10, 100, and 1,000 Hz, respectively. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

intracellular motion following PEF; most importantly, we observed a correlation between the persistence of cytoplasmic streaming and the degree of PEF-induced tissue damage. This finding suggested the hypothesis that cytoplasmic streaming plays a role in increasing the cellular damage at sufficiently low PEF frequencies. To directly test this hypothesis, CB was used to inhibit cytoplasmic streaming in onion epidermis prior to PEF treatment as described in the previous section, and the electrical conductivity of the onion epidermis was evaluated before and after PEF in order to calculate the electrical conductivity disintegration index Z^*. Since our previous work indicated that frequency plays a significant role, measurements were performed over a wide range of frequencies, from 0.1 Hz to 1 kHz.

First, we note that our microscopy observations of the control samples revealed streaming behavior qualitatively similar to our previous work[7], i.e., at high frequencies the cytoplasmic streaming stopped relatively quickly whereas at low frequencies it decayed more slowly. In contrast, the tissues treated with CB already had their cytoplasmic streaming inhibited (cf. Figure 4); in these samples only Brownian motion was observed during and after PEF.

The effect of CB on the conductivity disintegration index following PEF treatment at $E = 333$ V/cm, $n = 10$ is shown in Figure 5. Several trends are apparent. First, for all samples the PEF treatment invariably yielded a higher value of Z^* compared with the controls, and this observation is consistent with the preliminary experiments discussed under the section "Determination of PEF Parameters." The second key trend was that lower pulse frequencies ($f \leq 1$ Hz) yielded $Z^* \approx 0.5$–0.7, which is significantly higher ($P < 0.05$) than both the controls ($Z^* = 0$) and samples treated at higher pulse frequencies between 10 and 1,000 Hz ($Z^* \approx 0.1$–0.3). This result indicates that lower frequencies result in greater PEF efficacy (or tissue damage) than higher frequencies.

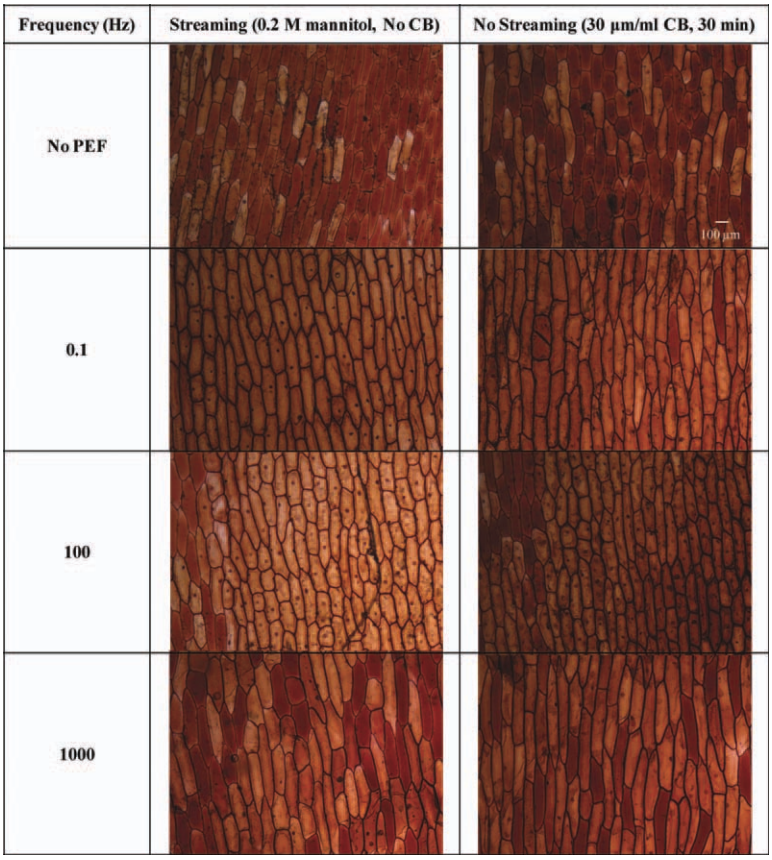

Figure 6. Representative photomicrographs of a cell viability assay demonstrating the effect of cytoplasmic streaming at different pulse frequencies on the integrity of onion epidermis.

All samples treated with PEF at $E = 333$ V/cm, $t_i = 100$ μs, $n = 10$ pulses (at least three replicates for each treatment). (a) No PEF (Control), (b) 0.1 Hz, (c) 100 Hz, (d) 1 kHz. Bright red cells are viable; pale pink cells are nonviable. Scale bar is 100 μm.

Similar behavior was observed in the previous studies with onion scales[7] and apple tissues[11], which are more complicated structures comprised of parenchyma and outer epidermal tissues and/or heterogeneous cell sizes. Despite the relatively simpler structure, onion epidermal tissues responded to PEF frequency in a fashion similar to onion scales, suggesting that the frequency dependence of PEF-induced cellular damage does not depend on the presence of parenchyma or cell and tissue heterogeneity.

The third and most important observation is the direct comparison of cells treated with CB to inhibit cytoplasmic streaming versus the controls. At high frequencies ($f \geq 1$ Hz), there is no statistically significant difference in Z^* between samples with or without cytoplasmic streaming ($P < 0.05$). In contrast, at $f = 0.1$ Hz the absence of cytoplasmic streaming resulted in a statistically significant ($P < 0.05$) reduction of Z^* from 0.68 to 0.55, i.e., a reduction of ~19%. In other words, by inhibiting cytoplasmic streaming, 19% fewer cells were permeabilized when PEF was applied at 0.1 Hz, and no statistically significant difference was observed at higher frequencies.

Effect of cytoplasmic streaming on cell viability: viability staining

Since an intact cell does not always remain viable[21], a viability staining technique was used to further assess the effect of cytoplasmic streaming on the efficacy of PEF treatment. We focused on the frequencies at $f = 0.1$, 100, and 1,000 Hz. These frequencies were selected because they yielded significant differences in onion tissue integrity after PEF. We used NR staining to distinguish viable cells from non-viable cells by the ability of viable cells to retain red stain. NR dye can easily diffuse through intact plasmalemma and tonoplast membranes into the vacuole, where it accumulates because of ionization of the dye in the low pH environment (e.g., pH 5–5.5) inside the intact vacuole.[7,45–48] Viable cells are indicated by a concentrated red area, while non-viable cells contain less dye because of the ruptured membranes. Figure 6 shows representative images of epidermal cells stained with NR following PEF at different frequencies for cells either treated or not treated with CB. The control samples (no PEF) contained mostly viable cells, i.e., the proportion of stained cells was ~80%. Although CB markedly reduced the speed of cytoplasmic streaming (cf. Figure 4), it did not induce any significant difference in cell viability ($P < 0.05$). In other words, the cells remained viable (as determined by NR staining), even though cytoplasmic streaming was inhibited.

In contrast, the cells treated by PEF exhibited markedly lower fractions of viable cells, consistent with the measurements of the conductivity disintegration index (cf. Figure 5). At 0.1 Hz, few viable cells were observed, whereas slightly more viable cells were observed at the higher frequencies. These trends are captured more quantitatively in Figure 7, which shows the percentage of stained (viable) cells measured over three trial replicates for each condition. For $f = 0.1$ Hz, the percentage of stained cells for samples with cytoplasmic streaming was statistically significantly lower than that for samples without cytoplasmic streaming ($P < 0.05$). In the absence of cytoplasmic streaming, the percentage of stained cells decreased from ~5.6% to only 0.3%. A comparatively larger number of cells remained viable in onion tissues treated at higher frequencies. For $f = 100$ and 1,000 Hz, samples with and without cytoplasmic streaming had ~5% and 21% viable cells, respectively. Notably, at these higher frequencies there is no statistically significant difference between cells with or without cytoplasmic streaming; in other words, the presence or absence of cytoplasmic streaming had no observable effect on the efficacy of PEF at higher frequencies.

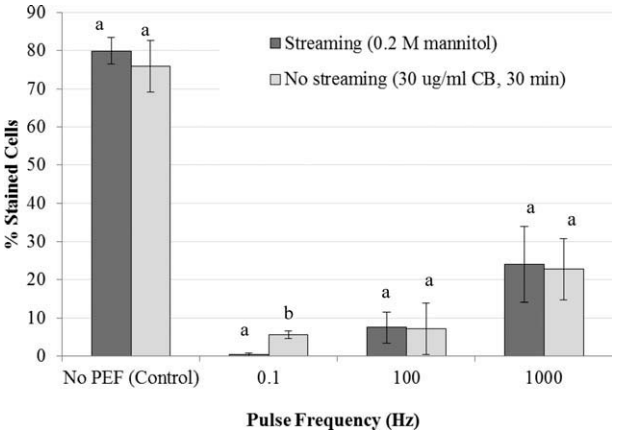

Figure 7. Effect of cytoplasmic streaming on the viability of onion epidermal cells treated with PEF at different pulse frequencies.

At least three replicates were done for each treatment; error bars represent one standard deviation of the mean. Quantification of cell viability was expressed as a percentage of stained cells using a cell count method. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$). Statistical comparisons were done separately for each pulse frequency.

Discussion and Conclusions

The primary hypothesis tested here is that cytoplasmic streaming enhances the efficacy of PEF at low pulse frequencies. Our direct video observations confirmed that Cytochalasin B effectively halted cytoplasmic streaming inside onion epidermal cells within a few minutes of application. Subsequent treatment of the epidermis by PEF yielded no statistically significant difference between control samples and samples treated with Cytochalasin B, provided the pulse frequency was 1 Hz or larger. In contrast, the neutral red viability assay and the conductivity disintegration index measurements both indicated that samples treated with Cytochalasin B were less affected by PEF at 0.1 Hz. The results strongly suggest that cytoplasmic streaming facilitates cell rupture at frequencies less than 1 Hz.

Another key observation, however, is that epidermal cells without cytoplasmic streaming still exhibited more damage at lower frequencies than cells either with or without cytoplasmic streaming at high frequencies (cf. Figures 5 and 7). This observation suggests that cytoplasmic streaming is not solely responsible for the increased PEF efficacy at lower frequencies. As discussed previously, in the context of onion scales[7], a mechanism that accounts for the increased efficacy at lower frequencies involves the movement of relatively high-conductivity fluid, originating inside the cell in the cytoplasm, through pores in the plasma membrane induced by PEF and into the lower conductivity extracellular space or apoplasm. By increasing the conductivity of the extracellular fluid around ruptured cells, less 'restrictive' paths are established for the next electric pulse to travel along and reach intact cells. Two transport processes help move conductive salt species through the cell wall: diffusion and

convection. Notably, the characteristic time scales for diffusion and convection are comparable at 0.1 and 0.05 Hz, respectively[7], suggesting that both processes have sufficient time to transport appreciable amounts of salt at PEF frequencies below 1 Hz. In other words, high frequencies are less effective because there isn't enough time for the more conductive fluid to move into the extracellular space. In this work, we effectively removed the influence of convection by inhibiting cytoplasmic streaming, but frequencies below 1 Hz still yielded a significant increase in cell damage compared to higher frequencies. This finding suggests that diffusive transport following membrane rupture plays a key role in the increased PEF efficacy at lower frequencies, whereas convective transport associated with cytoplasmic streaming plays a secondary but statistically significant role.

Although we only tested onion epidermis cells here, previous observations suggest that cytoplasmic streaming could play a similar role in other plant tissues. Specifically, the effect of PEF frequency observed here in onion epidermis is qualitatively similar to previous observations in apple tissue[11] and onion scales[7]; in both studies frequencies below 1 Hz caused significantly more damage. Presumably the same interplay of diffusive and convective transport is operative. The key practical implication is that PEF treatment will be most effective when cytoplasmic streaming is most active, i.e., with freshly prepared plant tissues. Long delays after sample preparation may reduce the cytoplasmic streaming speed, which may lead to a reduction in PEF efficacy. For onion epidermis, it took ~6 hours after sample preparation to observe cessation of cytoplasmic streaming, suggesting that PEF treatment is best done in a much shorter time period. The results presented here suggest the need to consider the influence of cytoplasmic streaming when performing low frequency PEF treatments of plant tissues.

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