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Sub-microsecond, intense pulsed electric field applications to cells show specificity of effects

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Abstract

Application of sub-microsecond duration (60–300 ns), intense (15–60 kV/cm) pulsed electric fields (sm/i-PEF) to six types of human cells was examined for its effects on individual cell surface membrane permeability and membrane potential. With short (60 ns) pulses, increasing percentages of Jurkat cells showed propidium iodide (PI) uptake at progressively shorter post-pulse times as the pulse train increased from 1 to 10 sequential pulses, while human blood polymorphonuclear leukocytes (PMN) were unresponsive to these short pulses regardless of train size. With 300 ns pulses, a similar pattern (increasing percentages of cells taking up PI, and progressively shorter times of onset after pulse applications as pulse train size increased) was seen with both Jurkat cells and PMN, but the patterns for both effects were different. Jurkat cell size did not appear to influence the responsiveness of this cell type. Comparisons of sm/i-PEF-induced PI uptake by human monocyte-derived macrophages vs. aged human mononuclear cells, human trunk skin (HTS) cells vs. fresh human mononuclear cells and human macrophages vs. HTS cells showed similar overall effects, but with differences between the patterns for each cell type compared (except the macrophages vs. HTS cells comparison). Application of sm/i-PEFs also caused different patterns of membrane potential loss in Jurkat cells vs. PMN. Jurkat cells developed significant decreases in t heir membrane potential only following the highest intensity pulse applications examined, i.e., 300 ns, 60 kV/cm ×5, while PMN showed this effect over the entire range of pulse intensities (300 ns, 15–60 kV/cm, ×5) applied. These data indicate that sm/i-PEF applications can have "specificity" (i.e., achieve different levels of effect in different cell types), that cell size does not appear to be the major factor determining sm/i-PEF effects in either Jurkat cells or PMN, that heterogeneous sm/i-PEF effects on cells tend to become homogeneous with increasing pulse train size, and that specificity of sm/i-PEF applications effects can occur at either end of the sm/i-PEF intensity spectrum examined.

Keywords: Pulsed electric field; Fluorescence microsocopy; Specificity; Propidium; Iodide; Membrane potential

1. Introduction

Application of sub-microsecond duration, intense pulsed electric fields (sm/i-PEF) to cells can cause selective permeabilization of intracellular membranes without immediate electroporation effects at the surface membrane [1]. The biological results of these pulse applications appear to include induction of programmed cell death in some cell types [2]. However, failure to elicit the characteristic biochemical changes of apoptosis in every cell type exposed to the same pulse conditions suggested that pulse applications might have heterogeneous rather than homogeneous effects from one cell type to another, i.e., that cellular specificity of pulse effects might exist. Because potential biomedical applications for sm/i-PEF might involve tissue or cell suspensions containing multiple cell types, determining whether sm/i-PEF applications have specificity of effect from one cell type to another, and which cellular characteristics might influence this specificity were of interest to us. Therefore, the following studies were performed.

2. Materials and methods

2.1. Materials

Hanks Balanced Salt Solution without Ca^{++} or Mg^{++} (HBSSw/o) was purchased from Fisher (Pittsburgh, PA).

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Propidium iodide (PI), 3,3′-dipentyloxacarbocyanine iodide ($DiOC_5(3)$) and carboxyfluorescein diacetate succinimidyl ester (CFDA) were purchased from Molecular Probes, (Eugene, OR).

2.2. Cell preparations

Human polymorphonuclear leukocytes (PMN) were prepared from heparinized human blood by Hypaque-ficoll step gradient centrifugation, followed by dextran sedimentation and hypotonic lysis [3]. To produce human mononuclear leukocyte preparations, mononuclear cell (MN) fractions collected from the Hypaque-ficoll step gradients used for PMN purification were washed once in HBSSw/o and used. To produce monocyte-derived macrophages, these MN fractions were resuspended in RPMI 1640 medium with 5% autologous sera and penicillin, streptomycin and glutamine, placed in sterile teflon jars and incubated for 10–30 days at 37 °C with 5% CO_2. Jurkat T cells and human trunk skin (HTS) cells were originally obtained from the American Type Culture Collection. Jurkat cells were maintained in culture using RPMI 1640 with added 10% fetal bovine sera, penicillin, streptomycin and glutamine, and were passed every 3–5 days. HTS cells were maintained in culture using DMEM with added 10% fetal bovine serum, penicillin, streptomycin, and sodium bicarbonate, fed twice per week and passed every 3–4 weeks. When used in experiments, Jurkat cells and HTS cells were washed once in HBSSw/o, then resuspended in HBSSw/o at the desired cell concentration.

2.3. Microscope sm/i-PEF device

The microscope system was designed/built to facilitate examination of the temporal development of sm/i-PEF effects in living cells (Fig. 1). With this system, a cell chamber is examined at 400× magnification using an Olympus IX70 inverted microscope (Olympus America, Melville, NY). The chamber is comprised of a 51 × 76 mm glass microscope slide with two 0.1-mm-thick stainless steel electrodes attached to the slide surface with silicone adhesive. The two electrodes are placed 0.33 mm apart to form a channel 5 mm long.

Following placement of the cell suspension (40–70 μl) in this channel, a standard 0.17-mm-thick glass cover slip is laid over the electrode gap, the channel is aligned with the microscope light path and solid copper contacts attached to a high voltage power supply are laid on the stainless steel electrodes.

To produce 60 and 300 ns pulses, a Blumlein Pulse Forming Network (PFN) was switched with a pressurized spark gap. The Blumlein PFN consists of two 50 Ω coaxial cables, which give a total impedance, Z, of 100 Ω. The energy stored in the cables is transferred into the matched load in the form of a rectangular power pulse, where the pulse duration is determined by the cable length and the

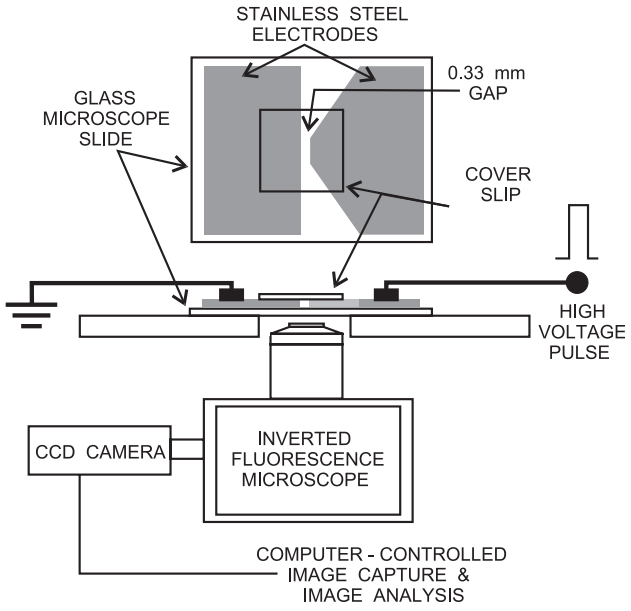

Fig. 1. Cartoon illustrating the experimental set-up of pulsing, microscopy and image recording systems.

speed of electromagnetic waves in the dielectric of the cable. For the matched condition to be achieved, the load (which in this case is the cell suspension between the electrodes) must have a resistance identical to the source impedance, Z. The spark gap, which serves as an electrical switch, has a closing time of < 10 ns. With this switch, the pulse generator produces electrical pulses with amplitude jitter < 5%. The maximum electric field that can be applied to the chamber (load) with the present design is given by the ratio of the maximum voltage (V_{max}) which can be applied to the load to the distance, d, between the electrodes (V_{max}/d). For our system, the maximum voltage was 2 kV, resulting in a maximum electric field of 60 kV/cm, with the hold-off voltage of the cable connectors the limiting factor.

Merlin software (LSR, Cambridge, England, UK) was used to control image capture before and after pulse applications, and for fluorescence filter management. To minimize fluorescence quenching and heating of the specimen, ultraviolet illumination of the specimen occurred only during image capture (1.6 s duration) which was triggered at 10-s intervals (11.6 s = 1 frame). Pulse applications were started at either frame 7 or 30 depending on the experiment. The effects of pulses on cells were recorded using a low-light, computer-controlled CCD camera (Olympix FKI300, Olympus America). The maximum recording speed of the camera (10 ms/frame) is long compared to the pulse duration of the applied electric fields but sufficient for use since the biological processes of interest occur on a time scale much longer than the shutter speed.

For PI experiments, a white light image rather than of a fluorescent image was acquired at the start of each experiment, immediately after the pulse series, at 10-min intervals

throughout the experiment, and then at the end of the experiment.

For $DiOC_5(3)$ experiments, white light images were taken at the start of the experiment, immediately before the pulse, 5 min later, and at the end of the experiment. White light illumination intensity and CCD camera sensitivity were adjusted so that the image gray scale sensitivity for both fluorescence and white light images were in the 50–350 unit range. At the completion of each experiment, image analysis of the fluorescent images was performed as described.

2.4. PI labeling experiments

Cells (PMN, MN, macrophages, Jurkat cells or fibroblasts as mixtures of different cell types) were resuspended in HBSSw/o (7.5×10^6 cells/ml) containing 15 μM PI. When mixtures of cells with similar dimensions (e.g., PMN and Jurkat cells) were used, one cell type was labeled with CFDA (3 μM) according to the manufacturer's instructions prior to mixing of the two cell types. CFDA is a cell-permeable, amine-reactive fluorescent label that yields long-term (days) labeling of cytosol and was used to allow identification of similarly sized cells as to their type. At the concentration used for labeling, CFDA fluorescence could only be seen when maximal camera sensitivity settings were used, and was not visible at the gray scale settings used to detect PI uptake by cells. Seventy-five microliters of this cell suspension was placed in the microscope cuvette and examined (excitation 340 nm, emission 500 nm). Computer-controlled image capture was triggered every 11.6 s (1 "frame"), and at frame 7, sm/i-PEF application(s) were made. When pulse trains of 5 or 10 were applied, individual pulses were applied approximately every 10 s. In most instances, image recording as continued for a total of 158 "frames" (30 min). For analysis, individual cells on the video image were identified and the gray scale intensity of the central part of each cell/nucleus (using the software's default detection area size, which was typically approximately 1/3–1/2 of the two-dimensional nucleus area) was recorded over the entire length of the experiment. PI uptake for a single cell was defined as >10 units increase in gray scale intensity over the background gray scale value for the experiment. The percentage of cells showing PI uptake, and the timing of onset of PI uptake were used as measures of sm/i-PEF effects.

2.5. Jurkat sizing experiments

In five experiments utilizing 300 ns, 60 kV/cm pulses applied × 1, × 5 or × 10, PI uptake in "small" and "large" Jurkat cells were compared. Jurkat cells are heterogeneous in size (diameters 7–20 μm): Jurkat cells were judged as "small" if their vertical axis was < 10.8 μm based on visual comparison with a length standard, and were judged as "large" if their vertical axis was >15.8 μm based on visual comparison with a length standard.

2.6. $DiOC_5(3)$ experiments

Cells (PMN or Jurkat) were divided into aliquots containing approximately 7.5×10^6 cells in HBSSw/o and labeled with 50 nM $DiOC_5(3)$ for 5 min at room temperature [4]. $DiOC_5(3)$ is a slow-response membrane potential sensitive dye that has been used extensively to measure membrane potential in many cell types: loss of $DiOC_5(3)$ fluorescence correlates with loss of membrane potential [4]. After this incubation, cells were placed in the microscope cuvette and fluorescent images (excitation 465 nm, emission 500 nm) were captured every 11.6 s over 20 min. Applications of sm/i-PEFs were started at frame 30 and five pulses were applied, each pulse separated by approximately 10 s. For analysis of images, the gray scale intensity of individual cells (obtained using detection areas adjusted to include the entire cell) were measured throughout the course of the experiment. In control conditions, $DiOC_5(3)$ fluorescence progressively diminished across the 15 min duration of each experiment. Therefore, a control condition was included in each experiment in which cells were visualized for 15 min without sm/i-PEF applications. Curves of gray scale changes vs. time for each cell in a microscopic field were averaged to yield a single curve representative of that experimental condition, and this curve was normalized by expressing the maximum gray scale value observed as 100% of maximal fluorescence. The "normalized" representative curves were meaned across experiments to produce the curves that reflected the effects of specific sm/i-PEF conditions on $DiOC_5(3)$ fluorescence. These average "normalized" curves were compared by calculating the mean ± SE area under the curve between frames 30 and 50 and comparing these by *t*-test.

3. Results

3.1. Polymorphonuclear leukocytes vs. Jurkat cell PI uptake experiments

Preparations of fresh human PMN contained few cells with spontaneous PI uptake. In contrast, Jurkat cell preparations always contained a few dead cells (i.e., showing spontaneous PI uptake), which allowed inclusion of one or more of these cells in the microscope field as positive controls. Application of sm/i-PEF of varying intensities and pulse train sizes to mixtures of PMN and Jurkat cells resulted in heterogeneous PI uptake responses in the two cell types (Table 1). Application of 60 ns, 60 kV/cm pulses to PMN in single, 5 and 10 pulse trains, did not result in PI uptake by these cells (Table 1). For Jurkat cells in these mixed cell preparations, similar results were only obtained for single pulse applications. For 5 and 10 pulse trains, considerable percentages of cells took up PI and these percentages increased with increasing pulse train size. The median time of onset for Jurkat cell PI uptake following 60

Table 1
Percentages of human cell types showing PI uptake following sm/i-PEF applications

	Mean ± SE percentage of PI positive cells (*n*)		
	1 Pulse	5 Pulses	10 Pulses
60 ns, 60 kV/cm			
Human PMN	0 ± 0 (8)	0 ± 0 (2)*	0 ± 0 (8)*
Jurkat cells	1 ± 1 (8)	30 ± 9 (5)	88 ± 4 (8)
300 ns, 60 kV/cm			
Human PMN	2 ± 1 (7)	63 ± 18 (5)*	72 ± 14 (7)
Jurkat cells	75 ± 8 (7)	94 ± 4 (5)	98 ± 1 (7)
Human macrophages	8 ± 8 (5)	27 ± 11 (6)*	60 ± 10 (6)
Aged human MN	14 ± 4 (5)	74 ± 11 (6)	80 ± 11 (6)
Fresh human MN	26 ± 8 (4)	63 ± 3 (4)	82 ± 6 (4)
HTS cells	2 ± 2 (4)*	12 ± 6 (4)*	56 ± 19 (4)
Human macrophages	5 ± 3 (4)	54 ± 21 (4)	66 ± 22 (4)
HTS cells	13 ± 13 (4)	0 ± 0 (4)*	45 ± 21 (4)

Data shown correspond to timing of onset of PI uptake shown in Table 2.

ns, 60 kV/cm pulses was noted to increase with decreasing pulse train size (Table 2): following 10 pulses, median time for PI uptake was 8 min after pulsing, while after six pulses, the median time to PI uptake increased to 16 min after pulsing. The fact that very few cells took up PI after a single pulse application might therefore be due to the limited period of observation after pulsing (approximately 30 min) in our experiments.

Application of 300 ns, 60 kV/cm pulses to PMN/Jurkat cell mixtures in single, 5 or 10 pulse trains resulted in PI uptake by both cell types (Table 1). However, the percentages of each cell type taking up PI were significantly different for single pulses (Fig. 2). Both PMN and Jurkat cell uptake of PI showed a dose–response effect with pulse train size, although the percentages of each cell type taking up PI differed under the same pulse conditions. The median time of onset for PI uptake at 300 ns, 60 kV/cm was also different for the two cell types (Table 2). Taken together, these data demonstrate that 60 ns pulses result in delayed PI uptake in Jurkat cells, and do not induce PI uptake by PMN, while 300 ns pulses induce PI uptake in both cell types, but with different percentages of cells affected and different timings of onset for PI uptake. For both cell types, greater and less disparate effects are achieved when pulses are delivered in trains.

Human PMN consistently have diameters of 7–10 μm, while Jurkat cells show a larger range of cell sizes: a small percentage of Jurkat cells approximate the size of PMN, most have diameters about 50% larger than PMN, and occasional cells are three to five times larger than PMN. To examine whether cell size might affect sm/i-PEF induced PI uptake, the percentages of Jurkat cells taking up PI and the timing of their PI uptake by small (< 10.8 μm) vs. large (>15.8 μm) diameter Jurkat cells were compared in five separate experiments (300 ns, 60 kV/cm pulses). As shown in Table 3, no striking effect of cell size was observed, suggesting that cell size alone was unlikely to have a major influence on sm/i-PEF induced PI uptake.

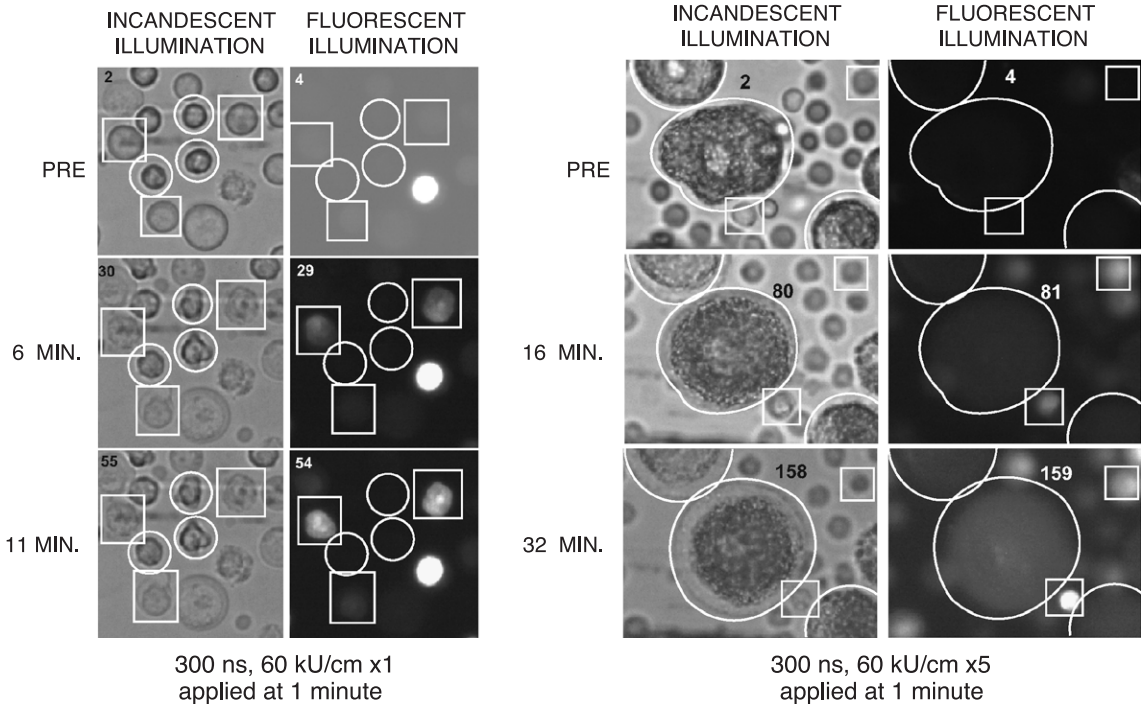

Fig. 2. Examples of differential PI uptake observed in PMN vs. Jurkat cells (left) and mononuclear cells vs. macrophages (right) exposed to the sm/i-PEF applications noted.

Table 2
Median time of onset for PI uptake by different human cell types following sm/i-PEF applications

	Median time of onset (minutes) after:		
	1 Pulse	5 Pulses	10 Pulses
60 ns, 60 kV/cm			
Human PMN	0	0	0
Jurkat cells	qns*	16	8
300 ns, 60 kV/cm			
Human PMN	qns	9	9
Jurkat cells	8	1	1
Human macrophages	qns	4	3
Aged human MN	10	1	1
Fresh human MN	5	2	1
HTS cells	qns	qns	1
Human macrophages	qns	2	1
HTS cells	qns	qns	1

Data shown correspond to percentages of cells taking up PI shown in Table 1.
*qns—too few cells taking up PI to estimate the median time of onset.

3.2. Human macrophage vs. aged human mononuclear cell PI uptake

Human monocyte cell fractions were isolated and the monocytes contained in them were allowed to differentiate for 10–25 days. When used experimentally, these cultures contained a mixture of macrophages plus "aged" undifferentiated mononuclear cells (lymphocytes and undifferentiated monocytes) that remained in the culture.

Monocyte-derived macrophages were identified by their size, which is typically 5–10 × larger than the aged non-macrophage residual cells remaining in the aged MN cell cultures. Application of sm/i-PEFs to mixed macrophage/aged MN cell preparations resulted in PI uptake by both cell types (Table 1, Fig. 2), with increasing percentages of cells taking up the dye as pulse train size increased. Comparison of the patterns seen with macrophages and aged MN cells showed that macrophages were less prone to sm/i-PEF induced PI uptake than aged non-macrophage mononuclear cells following sm/i-PEF applications. This demonstration of specificity was considered to suggest that differentiation state, aging or cell type might influence sm/i-PEF induced permeability to PI.

The differences in time of onset for PI uptake were less striking for this cell pair (Table 2). While PI uptake by was minimal for macrophages after a single pulse, aged MN had a median onset of PI uptake of 10 min after one pulse. With larger pulse trains, median onset of macrophage PI uptake occurred 4 min (5 pulses) and 3 min (10 pulses), while median onset of PI uptake by aged MN cells shortened to 1 min after both 5 and 10 pulse exposures. These data suggest that macrophages are less susceptible to the immediate effects of the multiple pulses compared to aged MN.

3.3. Fresh human mononuclear cell vs. human trunk skin cell PI uptake

In four experiments, fresh human MN were combined with HTS cells and exposed to sm/i-PEF applications. The HTS cells are at least 10 × larger then fresh MN so the two populations could be easily identified without CFDA labeling. Application of 300 ns, 60 kV/cm pulses to these cell mixtures resulted in increasing percentages of both cell types taking up PI as a function of pulse train size (Table 1). Similar to the results seen in the macrophage vs. aged MN cell comparison, the larger HTS cells were less prone to sm/i-PEF induced PI uptake than fresh MN cells after one or five pulses. Unlike macrophage vs. aged MN, the median time of PI onset for fresh MN became progressively shorter as the pulse train size increased (Table 2). For HTS cells, because PI uptake after one or five pulses was minimal, the time of onset for PI uptake for these conditions could not be addressed. However, after 10 pulses, the median time of onset was the same as observed with the fresh MN cells after 10 pulses: 1 min after pulsing.

3.4. Human macrophage vs. human trunk skin cell PI uptake

In four experiments, human macrophages were combined with HTS cells and exposed to sm/i-PEF applications. Both macrophages and HTS cells are large cells, with the HTS cells somewhat larger than the macrophages. Application of sm/i-PEFs to mixtures of these cells resulted in increasing percentages of both cell types taking up PI as a function of pulse train size (Table 1), with a significantly larger percentage of macrophages taking up dye after five pulses. For these experiments, only the median time of onset for PI uptake following 10 pulses could be compared, and it was the same (1 min after pulsing) for both cell types.

3.5. sm/i-PEF effects on human polymorphonuclear leukocyte and Jurkat cell membrane potential

Use of mixtures of PMN and Jurkat cells for experiments examining sm/i-PEF effects on membrane potential were

Table 3
Effects of Jurkat cell size on sm/i-PEF-induced PI uptake

Pulse train size	Cell size	Number (+)	Time at onset of PI uptake (minutes)			
		Total	<5	5–8	>8–14	>14
1	large	12/12	4	2	5	1
	small	12/15	2	2	8	0
5	large	11/12	11	0	0	0
	small	19/19	19	0	0	0
10	large	3/3	3	0	0	0
	small	17/17	17	0	0	0

Data shown are numbers of cells taking up PI following pulsing of 300 ns, 60 kV/cm ×1, ×5 or ×10.

not possible: at the excitation/emission wavelengths used to visualize $DiOC_5(3)$, CFDA had undetectable fluorescence. Therefore, PMN and Jurkat cell preparations were examined separately. Preliminary experiments demonstrated that single pulse applications had little acute effect on membrane potential (Control AUC: 15.9 ± 0.6% × frame (p × f) units (n = 3) vs. 300 ns, 60 kV/cm AUC: 15.4 ± 0.2 p × f units (n = 3)), leading to experiments in which pulse trains of five were used and electric field intensity was varied across 15–60 kV/cm. Jurkat cells showed significant loss of their membrane potential over the 4 min (frames 30–50) following initiation of pulsing only for the highest intensity electric field applications (Fig. 3, below). In contrast, PMN showed significant decreases in their membrane potential following all intensities of sm/i-PEF applications tested, 15–60 kV/cm. To assure that the unusual pattern of membrane depolarization observed in PMN was not simply due to surface membrane permeabilization-induced cell injury, the percentage of PMN taking up PI after 15, 30, 45, and 60 kV/cm exposures (each × 5) was determined: no pulse 0 ± 0%; 15 kV/cm 0 ± 0%; 30 kV/cm 43 ± 19%; 45 kV/cm 56 ± 23%; 60 kV/cm 96 ± 3%, all (n) = 3. Thus, significant decreases in PMN membrane potential occurred after sm/i-PEF applications (e.g., 15 kV/cm) that did not cause surface membrane permeabilization.

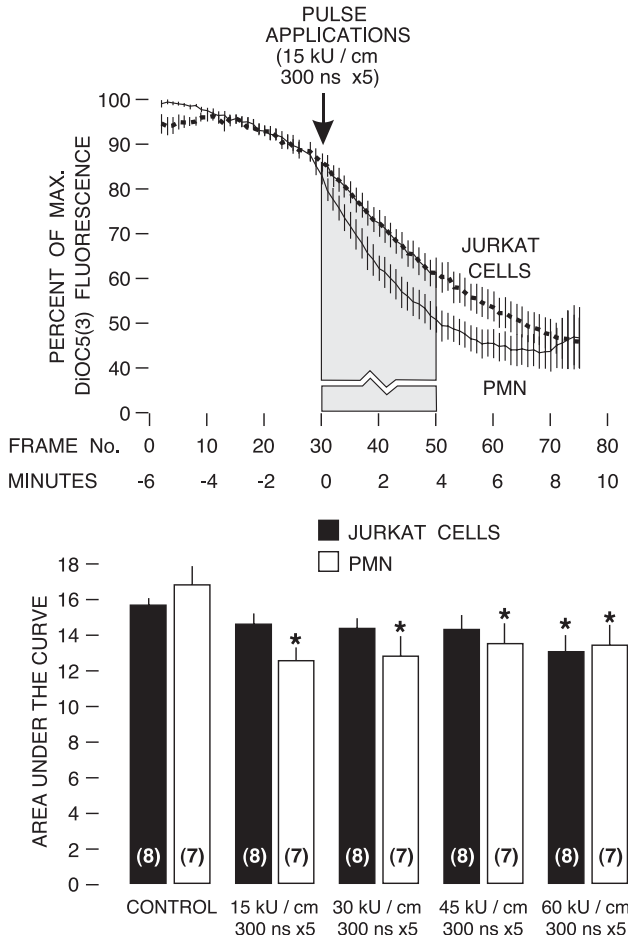

Fig. 3. Effects of sm/i-PEF on membrane potential of Jurkat cells and PMN. Top panel: $DiOC_5(3)$ fluorescence patterns of Jurkat cells (dotted line) and PMN (fine solid line) following sm/i-PEF applications (300 ns, 15 kV/cm, ×5) starting at frame 30. Data shown are mean ± SE values for $DiOC_5(3)$ fluorescence, shown as percentage of the maximal fluorescence value observed in each run. Grey stippling identifies region used to calculate the "area under the curve" data shown in lower panel. Lower panel: Mean ± SE area under the $DiOC_5(3)$ fluorescence curve for Jurkat cells (closed bars) and PMN (open bars) following sm/i-PEF applications identified at the base of each pair of bars. Asterisks indicate $p < 0.05$ vs. control.

4. Discussion

Recent reports [1,2,5,6] on the effects of sub-microsecond, high-intensity pulsed electric field applications to living cells indicate that with increasingly shorter pulses, these electric field applications should [6] and do [1,2] progressively focus their effects on the interior of the cell, rather than on the surface membrane as occurs with longer (multi-microsecond) pulsed field applications. In these reports, the effects of sm/i-PEF applications in several cell types have been described, but no comparisons of sm/i-PEF effects from one cell type to another have been made. In the process of examining the effects of these pulses on human eosinophils [1], we noted that PMN contaminating the eosinophil preparations did not appear to be affected by sm/i-PEF applications as the eosinophils were, despite the close differentiational relationship between the two cell types. Our interpretation of this was that "specificity" of effects from sm/i-PEF applications might be occurring, and the studies reported here were performed to determine whether specificity of sm/i-PEF effects occurs, and if so, which factors might affect the specificity.

It is generally accepted that PI uptake by cells following multi-microsecond duration pulsed electric field applications is the result of electroporation of the surface membrane, and it occurs promptly (seconds to minutes) following pulse application. With manipulation of the temperature, composition of the suspending medium used (cooling, low conductivity and low sodium content) and intensity (moderate) of the electric fields used, the duration of increased permeability of the surface membrane can be prolonged and the effect can be kept non-lethal. In theory, the mechanism by which PI uptake occurs following short pulses is different from the mechanism of classical electroporation. Short pulse effects are due to increased charge deposition at intracellular membranes (relative to surface membranes) [6], which preferentially affects/alters the intracellular membranes—an effect termed "intracellular electromanipulation" [1]. Experimentally, onset of PI uptake in Jurkat cells after single short (sub-microsecond duration) pulses becomes progressively delayed compared to the onset following single long (microsecond duration) pulses, concretely differentiating

the effects of short vs. long, i.e., classical ''electroporation'', pulses [7].

In initial experiments, PI uptake following short pulses was assessed in mixtures of two different cell types: Jurkat cells (T cell leukemia) and PMN (normal, terminally differentiated phagocytic cells isolated fresh from human blood). These studies illustrated that with respect to induction of permeability to PI, the percentages of cells taking up PI (Table 1) and the timing of onset for PI uptake (Table 2), each cell type responded differently to sm/i-PEF applications, i.e., that there was a degree of specificity regarding pulse-induced effects. A second effect seen in these experiments was that with increasing pulse train size, the effects of sm/i-PEF applications tended to become homogeneous, suggesting that cumulative effects might be occurring as pulse train size increased.

Extension of this experimental approach to additional pairs of cell types, monocyte-derived macrophages vs. aged MN, HTS cells vs. fresh MN, monocyte derived macrophages vs. HTS cells, tended to demonstrate similar effects. PI uptake characteristics of different cell types following one to five pulses were often different, but with increasing pulse train size, these differences diminished. We interpret these results to indicate that when PI uptake is used as an indicator, ''specificity'' of sm/i-PEF effects can vary from one cell type to another, but that in most cases, the specificity tends to be lost as pulse train size is increased.

Maintenance of electrical polarization of the surface membrane (i.e., membrane potential) is a characteristic of most cell types, and for electrically excitable cells (e.g., neurons, cardiac conduction system cells), reversible alteration of membrane potential is a mechanism for propagation of signals from one cell to another. For all cell types, membrane potential is lost with cell death, but in some cells, including PMN, reversible loss/gain of membrane potential occurs as a physiological event associated with intracellular signal transduction following receptor–ligand binding [4]. Under resting conditions, PMN membrane potential is maintained via a complex balance between intracellular and extracellular ions, including Na^{+}, K^{+} and Ca^{++}. In PMN, depletion of intracellular Ca^{++} stores results in loss of membrane potential [8], and membrane potential modulates agonist-induced intracellular free Ca^{++} levels [9]. The effects of sm/i-PEF applications on surface membrane potential were of interest, therefore, because sm/i-PEF would be expected to hyperpolarize and depolarize surface membrane at opposite poles to some degree, and in addition, should alter intracellular membrane structure due to the bipolar voltage pulse generated across these membranes when the monopolar electric field pulse is applied [1]. When PMN and Jurkat cell membrane potential was examined following sm/i-PEF applications, it was seen that for Jurkat cells, only electric field applications of 60 kV/cm (conditions that resulted in widespread PI uptake with rapid onset in these cells) resulted in a significant drop in membrane potential over the 4 min following pulsing. When applied to PMN, however, very different responses were seen—every pulsing condition down to electric fields of 15 kV/cm resulted in significant loss of membrane potential. For the 60 kV/cm applications, a condition that causes approximately 65% of PMN to take up PI with median onset at 9 min (Tables 1 and 2), significant loss of membrane potential occurred over the 4 min following pulsing. This suggests that intracellular events that lead to surface membrane depolarization precede those that cause increased surface membrane permeability. That the lowest electric field intensity examined caused the same magnitude of membrane potential loss as seen following the most intense electric field applications is curious because this pulsing condition is below the threshold for initiating PI uptake by PMN. Such an effect might result from sm/i-PEF triggering of intracellular signaling that normally involve membrane depolarization. While studies to understand the basis for this sm/i-PEF effect in PMN are currently underway, this effect illustrates an additional aspect of sm/i-PEF specificity: that sm/i-PEF applications can show specificity of effects as their intensity either increases or decreases.

The basis for sm/i-PEF applications' specificity effects on PI uptake remain obscure. It is well known that electroporation with multi-microsecond pulses is dependent on cell size, an effect that has been successfully used to discriminate between cells of different sizes [10]. With sub-microsecond pulses, however, cell size does not seem to determine either the degree or timing of onset of surface membrane permeabilization. Our observations show that sm/i-PEF effects are not dependent on cell size. When the PI uptake characteristics of large vs. small Jurkat cells were compared (a strategy that allowed other potential contributors to specificity, e.g., differences in biochemical composition, cell type, intracellular structures or growth conditions, to be held constant), no difference was observed. Secondly, the size of PMN and Jurkat cells significantly overlap as noted above, and yet, dramatic differences in both PI uptake (following 60 ns pulses) and membrane potential changes (following 300 ns pulses) were documented in our experiments.

Whether a normal vs. transformed state of cells influences specificity is also of interest. Of the six human cell types examined in these experiments, Jurkat, a transformed T cell leukemia cell line was the most sensitive to sm/i-PEF induced PI uptake. Of the normal cell types examined, it appeared that the resistance to sm/i-PEF-induced PI uptake was roughly in the order of HTS cells>macrophages>fresh MN cells>PMN>aged MN cells, which at least for human mononuclear cells, raises the question of whether cellular differentiation state may alter responsiveness to sm/i-PEF exposures.

In summary, these experiments illustrate that cell responses to sm/i-PEF exposures, assessed by PI uptake, are delayed relative to the time of pulse application. We interpret

the temporal separation of membrane depolarization and PI uptake as consistent with intracellular effects, as expected for sub-microsecond pulses, that precede effects that lead to surface membrane permeability. These experiments illustrate that cell responses to sm/i-PEF exposures, based on either PI uptake or membrane depolarization responses, are inhomogeneous, i.e., demonstrate ''specificity'' at low pulse train sizes, and that as pulse train size increases, cellular responses become more homogeneous across different cell types. They also show that heterogeneity of cellular responses to sm/i-PEF can be based on pulse intensity as well as target cell type. These results suggest that sm/i-PEF may be ''tunable'', i.e., can have different effects on individual cell types depending on the pulse characteristics and train size used, which may allow their selective use to address a variety of biomedical problems and conditions.

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