

# Cell responses without receptors and ligands, using nanosecond pulsed electric fields (nsPEFs)

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The plasma membrane is a lipid bilayer that surrounds and shelters the living structural and metabolic systems within cells. That membrane is replete with transmembrane proteins with and without ligand binding sites, oligosaccharides, and glycolipids on the cell exterior. Information transfer across this structure is closely controlled to maintain homeostasis and regulate cell responses to external stimuli. The plasma membrane is contiguous with the endoplasmic reticulum (ER) and nuclear membranes. A number of proteins form ER-mitochondria junctions, allowing interorganelle communications, especially for calcium transport. Transport mechanisms across these membranes include nongated channels or pores; single-gated channels for ion transport; carrier molecules for facilitated diffusion; and pumps for active transport of ions and macromolecules. During the activation of these transport systems, “pores” are formed through protein structures that transiently connect the intracellular and extracellular milieu. These pores are nanoscale structures with diameters of 0.2–4.0 nm. However, there can also be maligned movements of molecules across the plasma membranes. *Staphylococcus aureus* protein  $\alpha$ -toxin and *Streptococcus pyogenes* protein streptolysin O both create pores that allow unsolicited molecular transfer across membranes that disrupts vital functions. Cytotoxic T-cells permeabilize the invading cell membranes with perforin, creating pores through which granzymes can induce apoptosis. These pores have a lumen of 5–30 nm with the majority at 13–20 nm.<sup>1</sup>

There are also nonbiological means to permeabilize plasma membranes that can be effective in delivery of drugs or nucleic acids into cells for basic biological or therapeutic purposes. In contrast to nature’s permeabilization of plasma membranes through proteins, these nonbiological methods permeabilize the lipid bilayer itself. Some methods include sonoporation (high frequency ultrasound and microbubbles), laser- or optical-transfection, and electroporation using electric fields to create “pores” or aqueous channels (there are also a number of other transfection methods that do not necessarily form pores in cell membranes).

Over 30 years ago, electric fields were shown to transiently permeabilize plasma membranes and to introduce deoxyribonucleic acid (DNA) into cells by electroporation.<sup>2</sup> A quarter of a century later, electroporation has been used in clinical trials with gene electrotherapy (GET) to deliver interleukin (IL)-12 to lesions in melanoma patients, showing safety and efficacy.<sup>3</sup> Another use of electroporation is to deliver impermeant cancer drugs, such as bleomycin, to tumors by electrochemotherapy (ECT).<sup>4,5</sup> Clinical applications for ECT have been demonstrated,<sup>6</sup> and ECT is now practiced clinically in

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several European Union countries.<sup>7</sup> Another method of cancer treatment using electric fields is to irrevocably permeabilize tumor cells by irreversible electroporation (IRE).<sup>8</sup> While GET, ECT, and IRE all use pulse durations in micro- to millisecond ranges, GET and ECT use electric fields of a few hundred V/cm; in contrast, IRE uses increased electric fields, up to 3 kV/cm. This creates cell permeabilization conditions that are irreversible, and cells are ablated and die, primarily by necrosis.

Another new electric field application in basic biology and medicine is pulsed power technology, which has been used for decades for military applications and pulsed power physics.<sup>9,10</sup> This approach makes greater use of high-voltage capacitors with fast discharge capabilities. These devices compress and immediately release high-power electrical pulses of extremely short duration into cells or tissues. In contrast to GET, ECT, and IRE, pulsed power delivers pulses of nanosecond duration, with higher intensity electric fields. These high power, low energy, nonthermal pulses are called nanosecond pulsed electric fields (nsPEFs) or nanoelectropulses. The pulses that are used have durations of 4–600 ns and electric fields of up to 60–80 kV/cm; however, electric fields as high as 300 kV/cm have been used, with pulse durations of 10 ns. By exploiting the variables of cell/tissue type and nsPEF conditions (pulse number, duration, intensity, and repetition rate), a range of biological responses can be observed. There is evidence that nsPEF-induced cell responses are different from the responses to electroporation.<sup>10–12</sup>

In contrast to conventional electroporation, under certain conditions, nsPEFs create small, propidium iodide (PI)-impermeable pores in plasma membranes and intracellular organelles, called nanopores.<sup>13,14</sup> The formation of high-density nanopores in all cell membranes is referred to as supraelctroporation.<sup>15,16</sup> These plasma membrane nanopores are long lasting (minutes) and exhibit complex ion channel-like conductance that is voltage-sensitive and inwardly rectifying. When these pores become PI-permeable, like conventional electroporation pores, they become highly conductive and nonrectifying. These nanopores are hypothesized to induce many nsPEF effects.<sup>14,16,17</sup>

NsPEF effects occur in the absence of ligands or receptors. NsPEFs have been shown to breach intracellular granules,<sup>9</sup> to mobilize Ca<sup>2+</sup> from the ER and through plasma membranes,<sup>18–20</sup> and to modulate intracellular Ca<sup>2+</sup> levels in chromaffin cells, mostly or exclusively through nanopores in the plasma membranes.<sup>21</sup> The mobilization of Ca<sup>2+</sup> induces secondary responses to nsPEFs and manipulates selected responses to changes in the intracellular Ca<sup>2+</sup>. In human

neutrophils, nsPEFs suppress spontaneous H<sub>2</sub>O<sub>2</sub> production and reversibly attenuate chemotaxis but do not affect phagocytosis, the least sophisticated neutrophil activity of the three functions.<sup>22</sup> The nsPEF-induced mobilization of Ca<sup>2+</sup> also activates human platelets-forming platelet gels,<sup>23</sup> induces action potentials in cardiomyocytes,<sup>24</sup> activates mitogen-activated protein kinase (MAP)<sup>25</sup> and adenosine monophosphate (AMP)<sup>26</sup> kinases, and induces a novel stress response.<sup>27</sup> NsPEFs induce calcium-dependent reactive oxygen species, which appear to require calcium entry through the plasma membrane pores.<sup>28</sup> Most recently, it has been shown that pulses of 10 ns duration can induce the plasma membrane permeabilization of giant unilamellar vesicles to small interfering ribonucleic acid (siRNA).<sup>29</sup> This provides yet another potential means to elicit cell responses without reliance on receptors and ligands.

NsPEFs also cause a rapid time- and electric field-dependent dissipation of the mitochondria membrane potential ( $\Delta\Psi_m$ ).<sup>30–32</sup> Interestingly, loss of  $\Delta\Psi_m$  requires an influx of Ca<sup>2+</sup> through the plasma membranes, which is necessary, but not sufficient to dissipate  $\Delta\Psi_m$  and induce cell death.<sup>11,33</sup> Since plasma membrane permeabilization is not Ca<sup>2+</sup>-dependent,<sup>17</sup> nsPEF-induced loss of  $\Delta\Psi_m$  is not likely due to nanopore formation in the inner mitochondria membranes, which would be expected based on the supraelctroporation hypothesis. Since most, if not all Ca<sup>2+</sup>-mediated mechanisms are regulated by proteins, the hypothesis that nsPEF effects are solely determined by membrane-charging events may require reconsideration. Given the close relationship between Ca<sup>2+</sup>-dependent losses of  $\Delta\Psi_m$  and viability,<sup>11,33</sup> it is likely that these events lead to mitochondria-mediated, intrinsic, caspase-dependent and -independent cell death, which has been observed in Jurkat cells.<sup>34</sup>

Other studies suggest that not all nsPEF effects are mediated by cell membrane nanopores. It has been shown that nsPEFs inhibit voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels,<sup>35</sup> and induce cytoskeletal breakdown and nuclear membrane abnormalities.<sup>36</sup> By unknown mechanisms, nsPEFs also increase the substructures of small nuclear ribonucleoprotein particles or intrachromatin granule clusters, suggesting the modification of pre-messenger RNA-splicing mechanisms.<sup>37</sup> However, it should be considered that some nsPEF effects are secondary to or require plasma membrane pore formation. NsPEF effects can be considered “supranatural.” Living beings are electric in nature, composed of cell and tissue batteries in muscles, nerves, and other tissues. The application of additional charges from nsPEFs to intracellular structures and plasma membranes certainly transcends the natural. Since

nsPEFs do not occur in nature, living organisms have not been under evolutionary pressure to develop responses to this stimulus. As expected, the responses to nsPEFs are distinct from those induced by previously known forms of cellular stresses.<sup>27</sup> In this context, the author's laboratory has focused on cell death mechanisms as a means to eliminate cancers. This role has been recently reviewed for nsPEFs, as an alternative or adjunct for surgical removal of cancer.<sup>12</sup> Any one or more of the nsPEF-induced cell responses presented above could directly or indirectly contribute to cell death. What seems clear is that when used at low repetition rates (1–2 Hz), nsPEF-induced cell death is not thermal nor immediate, but somewhat calculated and finally, default-driven. The research landscape that has most recently and surprisingly emerged and that is the focus of our present work, is the presence of a vaccine effect<sup>38</sup> and possible immune response<sup>39</sup> that materialize during/after nsPEF-induced tumor cell death.

## Disclosure

The author reports no conflicts of interest in this work.

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