2012

Subcellular Biological Effects of Nanosecond Pulsed Electric Fields

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Abstract. Membranes of biological cells can be charged by exposure to pulsed electric fields. After the potential difference across the barrier reaches critical values on the order of 1 V, pores will form. For moderate pulse parameters of duration and amplitude, the effect is limited to the outer cell membrane. With the exposure to nanosecond pulses of several tens of kilovolts per centimeter, a similar effect is also expected for subcellular membranes and structures. Cells will respond to the disruption by different biochemical processes. This offers possibilities for the development of novel medical therapies, the manipulation of cells and microbiological decontamination.

Morphologies and functions of cells and their constituents can be changed by exposures to electric fields. The response that is instigated depends on the strength of the electric field and the duration it is acting upon the target. Short stimuli (on the order of milliseconds and voltages of several tens of millivolts) that are imposed across the cell membrane open voltage-gated channels and, in this way, regulate the transport of ions such as potassium and sodium across the membrane (1-3). Extended exposures of several seconds or even minutes with several tens of volts per meter provide enough energy to either denaturate proteins or even cause thermal damage directly, i.e., burn tissues. Stimulations of the first kind are used in electrophysiological studies of action potentials, for example. The second type of exposure, with the goal of delivering energy to malignant tissues, is utilized in radio- or microwave ablation therapies (4, 5). Instead of these quasi-continuous exposures, temperatures that are no longer conducive to survival can also be achieved with a single pulsed electric field of short duration, if the field strength is sufficiently high. More interesting, however, are more specific responses that can be accomplished with short, pulsed electric field exposures that will not result in thermal damage, but are already considerably stronger than stimuli that would merely trigger a physiological reaction by membrane proteins. Biological effects that are instigated above this threshold are generally caused by an initial increase in the permeability of membranes. This effect is generally attributed to the formation of pores. Characteristics and dynamics, i.e., pore diameter, their distribution along the cell surface, and lifetime, are critically dependent on the pulse duration and magnitude of the applied electric fields (6-10). In addition, many applications take advantage of a cumulative effect on the membrane and rely on exposures with bursts of individual pulsed electric fields. Biological processes, and ultimately, the fate of a cell, depend on the possibility that these pores will reseal, and on the transport of substances across the membrane, either by migration or diffusion, while they are open (11-13). Consequently, the delicate balance of ion concentrations of sodium, potassium, chlorine, calcium and others will be disturbed. Moreover, large molecules, such as pharmaceutical agents and

genetic material, can pass this otherwise-impenetrable barrier (14, 15). After membrane integrity has been restored, these additions to the cytoplasm will then participate in the cell's biochemistry.

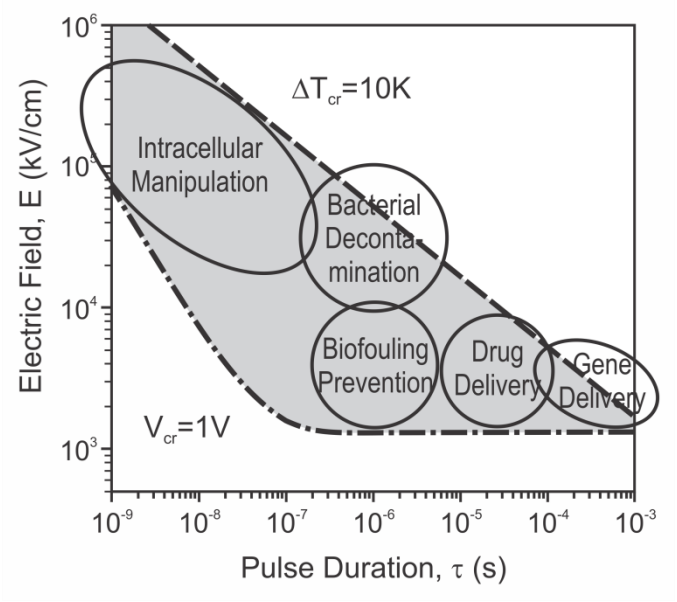

Figure 1. Parameter ranges for different applications of pulsed electric field exposures of cells. A potential difference across a critical voltage, V_{cr}, will result in the formation of pores, which initiate the subsequent response. For shorter pulse durations, increasingly higher voltages are required to reach the critical voltage during exposure as indicated by the dash-dotted line. (The calculation of the critical voltage, V_{cr}, is assuming a spherical cell of 10 µm, suspended in a medium of 70 Ωcm, equivalent to the conductivity of the cytoplasm.) The electrical energy applied will also heat cells, and thermal damage becomes more prevalent with increasing field strengths as indicated by the dashed line. (The calculation of the critical temperature increase, ΔT_{cr}, assumes an adiabatic heating of the cell volume filled with water.)

This offers intriguing possibilities for new medical therapies and novel biotechnological approaches. For example, can chemotherapeutic agents that are poorly membrane permeant, and consequently require large doses to be effective, now be efficiently delivered into tumor cells directly (14, 16). Another appealing application is the introduction of genetic material into cells to change their function and development. The method is currently being investigated for its potential as a vaccination against certain cancers, while the most prominent use of the technique is probably still in cloning (17, 18). Of course, if exposure parameters prevent restoration of membrane integrity, cells will die (13). Accordingly, pulsed electric fields have also been used successfully to ablate tissues and to inactivate microorganisms, particularly in liquids where most current applications focus on the decontamination of water and food, such as milk and juices (19-21). In fact, the killing of bacteria and yeast in water by this method was already reported by Sale and Hamilton in 1967, which is also commonly

assumed to be the first account of membrane damage by pulsed electric field (22, 23). The process was later described as 'electroporation' by Neumann (24). The name summarizes theories that explain the damage to the membrane and subsequent increase in permeability by the formation of pores under the influence of the electric field. When exposure conditions allow for membrane recovery, they may still have a temporary effect on microorganisms without necessarily killing them. For aquatic species, e.g., brine shrimp, a temporary inactivation or "stunning" is observed that might last several minutes but does not seem to permanently impair the organism's functions (25). Details of the mechanisms responsible for the observed transient inactivation of organisms of higher order are even less well studied and understood than for single cells. However, an obvious application of the method is the prevention of biofouling in water treatment facilities or the treatment of coolant water taken from ambient reservoirs (lakes and rivers) without the need for chemical solutions, and therefore, no environmental burden (26).

Basic Mechanisms of the Interaction of Pulsed Electric Fields and Cells

Different applications and the ranges for pulse durations and pulse amplitudes necessary to achieve a specific response not caused by thermal damage or by the physiological activation of membrane proteins, are shown in Figure 1. Most of these applications assume that an increase in membrane permeability is the underlying cause, and that this increase in permeability is primarily limited to the outer cell membrane. Subsequent biological responses are a result of the different processes that are set in motion by this damage. Whether pores form in the membrane depends on the voltage difference that can be achieved across the membrane during the exposure. Without damage, the cell membrane resembles a fairly good insulator. Charges (sodium, potassium and other ions) accumulate along this barrier during the application of an electric field, E, leading to a change in the transmembrane potential, V_{cell}. When the membrane potential change reaches a critical value ranging from several hundred millivolts to 1 volt, pores will form. (The value varies among cell types, but is close to 1 V for most cell membranes.) An extended exposure primarily provides the energy to increase pore size and number (27).

For a spherical cell with diameter, D, the charging characteristic at the poles of the cell (with respect to the direction of the field) is described by Equations 1 and 2 (28, 29). (The angular dependency of the induced membrane potentials is described by a cosine modulation of this maximum value – not shown.) Hereby, the charging time constant, τ_c, is again determined by the size of the cell, together with the specific membrane capacity, c_m, and the conductivities of the cytosol, ρ_c, and the cell suspension, ρ_a. Typical values for the resistivity of the cytoplasm for physiological cell solutions are on the order of 70 Ωcm, while values of 1 μF/cm^2 have been determined for the specific membrane capacitance (30, 31).

$$\left|\Delta V_{cell}(t)\right| = 1.5 \cdot E \cdot \frac{D}{2} \cdot \left(1 - \exp(-t / \tau_c)\right) \qquad (1)$$

$$\tau_c = \left(\rho_c + 0.5 \cdot \rho_a\right) \cdot c_m \cdot \frac{D}{2} \qquad (2)$$

More sophisticated analytical and numerical models have been developed, striving for a more accurate description of exposure conditions, different membrane structures and compositions (e.g., for bacteria), cell parameters and cell shapes (32-38). However, key observations can be sufficiently evaluated with this basic formula, particularly for spherical mammalian cells. According to Equations 1 and 2, for a cell with a diameter of 10 µm, an electric field of at least 1.34 kV/cm needs to be applied to achieve a change of membrane potential by 1 V in the steady state. Much higher electric fields are required for shorter exposure times (Figure 1). The analysis further shows that for a given electric field, larger cells reach critical transmembrane voltages faster than smaller cells. Accordingly, smaller microorganisms, such as bacteria and yeast, require exposures to higher electric fields to porate their membranes, and eventually inactivate them, than mammalian cells do for the transient permeabilisation required to introduce drugs.

With the charging of the outer cell membrane, a counterfield develops inside the cell, which effectively shields subcellular structures from further exposure to the applied field. Potential differences that are adjusting across the internal membranes of organelles, such as the nucleus, mitochondria, endoplasmic reticulum and others, during the evolution towards steady state conditions, can be described by Equation 3. (The derivation of the equation assumes a spherical organelle of diameter d in the center of a spherical cell. The organelle is exposed to an electric field, which is initially the same as the external field but is decreasing in the same manner as ΔV_{cell} is increasing. Again, the angular dependence is omitted.)

$$\left|\Delta V_{organelle}(t)\right| = (1.5)^2 \cdot E \cdot \frac{d}{2} \cdot \exp(-t/\tau_c) \cdot \frac{\tau_c}{\tau_c - \tau_o} \cdot \exp\left(\frac{t}{\tau_c} - \frac{t}{\tau_o}\right) \qquad (3)$$

The charging time constant for the organelle, τ_o, is determined by an expression similar to Equation 2, by replacing cell diameter, D, with organelle diameter, d, the conductivity of the cytoplasm, ρ_c, with the conductivity of the organelle content, ρ_o, and the ambient conductivity of the suspension, ρ_a, with the conductivity of the cytoplasm, ρ_a. (It is also assumed that the dielectric properties of all cellular membranes are identical to the characteristics of the outer cell membrane.) The comparison with the development of transmembrane voltages across the outer membrane shows that, for sufficiently high electric fields, subcellular membranes will also experience potential differences that are on the order of, or exceed, typical critical voltages. For a 5-µm diameter organelle (e.g., the nucleus) inside a 10-µm cell, and an exposure to a field of 10 kV/cm, the voltage changes after 5 ns across the membranes are $|\Delta V_{organelle}| = 0.93$ V and $|\Delta V_{cell}| = 0.68$ V, respectively. The example shows that subcellular membranes can in fact charge faster than the outer cell membrane. Accordingly, poration of organelle membranes and modifications of subcellular structures can be expected for strong applied electric fields. The example further shows that membranes are charged to critical voltages in only a few nanoseconds. Since the extent of the exposure, after critical values are met, primarily determines the further increase in pore density, short exposures on the order of, or shorter than, the charging time of the outer cell membrane

could have a more pronounced effect on organelles than on the cell membrane. Moreover, even a less extensive poration of subcellular membranes might be sufficient to trigger an irreversible biological response.

Equations 1-3 give a simplified description of the mechanisms following the application of an electric field. However, the approach can not account for many parameters of the exposure, such as actual subcellular geometries, membrane compositions, or non-linear events, such as the change in membrane conduction after pores have formed. Even the application of a field with an infinitely fast rise time is an idealization. More elaborate approaches have been employed to describe actual experimental conditions more accurately and gain further insight. Most interestingly, a more detailed analysis of the exposure to intense short pulsed electric fields with a fast rise time predicts that, in fact, the pores that can be generated under these conditions are different from pores that can be created by longer pulsed exposures of lower field strength as commonly used in electroporation techniques for the outer membrane. In particular, all membranes (outer cell membrane and organelle membranes) are uniformly porated within a few tens of nanoseconds into the exposure (7, 35, 39, 40). The number and density of pores is predicted to be several orders of magnitude higher when compared with electroporation pulses. However, pores are also expected to be much smaller, which would allow only small ions to pass through but not larger molecules (6, 41). Since many of the ions that are involved in the regulation of cellular functions still could permeate cellular membranes, cell functions are likely to be affected. As a result, intense ultrashort pulsed electric field exposures offer a method for intracellular manipulation of cells (Figure 1).

Intracellular Effects of Nanosecond Pulsed Electric Field Exposures

The theoretical analysis shows that exposures that primarily affect subcellular structures require pulsed electric fields with a duration that is short compared to the charging time of the cell envelope. (For a cell of 10 µm in diameter and resistivities for cell suspension and cytoplasm of 70 Ωcm, the charging time constant is 52.5 ns and the charging time is, accordingly, on the order of 200 ns.) In addition, the field strengths need to rise as fast as possible to amplitudes in the range of several tens of kilovolts per centimeter to expose also the subcellular space before it is shielded and critical voltages can no longer be achieved in the remaining field. These parameters can not be provided using conventional electronic circuits – at least not for the exposure of relevant cell or tissue volumes between electrodes at least a few millimeters apart. Alternatively, pulsed power technologies are employed (42). Basic circuits are based on pulse forming networks or pulse forming lines. When using a pressurized spark gap as the switching element, rise times of 1 ns have been realized for voltage pulses with amplitudes of 35 kV, corresponding to electric fields of 350 kV/cm across the electrode gap of 1 mm of a standard ‘electroporation-cuvette’ (43). The equipment allows the exposure of 100 µl of cell suspensions, which is sufficient for the post-exposure evaluation of biological and biochemical responses in particular. (For lower electric fields, larger volumes can be exposed.) Similar systems have also been used in *in vivo* experiments. In this case, high voltage pulses were usually delivered by needle electrodes instead of plane parallel electrodes (44-46). For the observation of early processes during or immediately after the exposure, microscope-mounted systems have been developed (47-49).

The fastest mechanism that has been observed so far after the exposure to nanosecond pulsed electric fields is the charging of the outer cell membrane (47). A fast voltage sensitive fluorescent dye was used, reflecting changes in the electric field across the membrane by characteristic changes in excitation and emission spectra. These changes were recorded with a temporal resolution of 5 ns and quantified to describe the development of the associated transmembrane potential. An example of the measurements is shown in Figure 2.

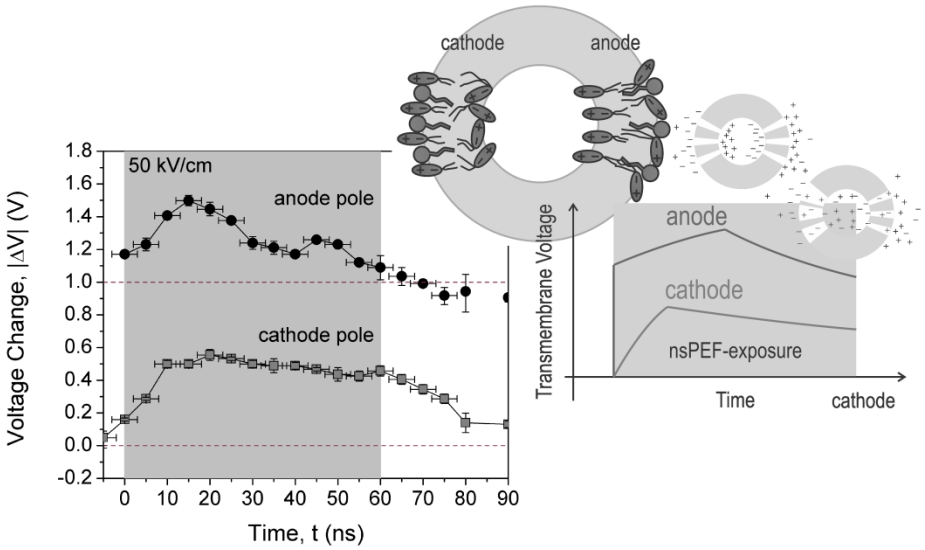

Figure 2. Transmembrane voltage changes of Jurkat cells (absolute values) for the exposure of 60 ns to an electric field of 50 kV/cm. Values at the anode pole jump to more than 1 V immediately. The difference in the values across anode and cathode might be accounted for by the alignment of phospholipid heads, which then affect the local electric field. Pores are gradually opening across the membrane already during exposure and after reaching a peak value of about 1.4 V allow for significant discharge currents.

The highest change in voltage (about 1.6 V) was observed at the anodic pole of the cell (the side facing the anode in a parallel plate exposure system). The change corresponds approximately to the values expected from theoretical models (50). However, a significant difference has been observed between the anode and cathode poles, with voltages that are 1 V lower at the cathode pole. Transmembrane voltages across the cathode side also develop more slowly than for the anode side, which suggests significant contributions to the potential difference across the membrane from dipole alignment. These dipoles are found in headgroups of phospholipids that are embedded in the membrane. (In addition dipole moments might also be induced by the electric field.) The restricted mobility of the molecules might account for the observed differences between hemispheres (51-53). The time at which the peak value in transmembrane potential is approached during the exposure depends on the amplitude of the applied electric field. After the peak voltage is reached, values start to decrease again. Apparently pores are starting to open across the membrane very early during the

exposure. Their continuous increase in number would allow for a limited migration of ions, which first impedes charging and eventually will lead to a reversal and discharge the membranes through these leaks at an increasing rate.

Due to the significance of the cell membrane as the interface for the cell to receive and process outside stimuli, and to the readily available methods to study these interactions, many studies have focused on the effects on the outer membrane (54-60). They have confirmed that the membrane initially becomes permeable for smaller ions only. However, for relatively long exposures and relatively high electric fields, it is possible that the membrane eventually becomes permeable for larger molecules (59, 60). Some experiments on the permeability for small ions show certain other characteristics, such as ion selectivity or preferences of ion movements that can not be explained by simple diffusion through holes in the cell envelope alone (57, 61). Likewise this model cannot sufficiently explain the observed translocation of phospatidylserine to the outer cell surface (62, 63). (The membrane protein is usually found exclusively on the inside of healthy cells and expressed on the outside only when cells undergo apoptosis.)

The reasonably good agreement between the measurements of transmembrane potential voltages induced across the outer membrane and theoretical predictions suggests that predictions for the poration of subcellular structures by nanosecond pulsed electric fields are reasonable as well. Unfortunately, measurements are not yet available on the development of transmembrane voltage changes across internal membranes. Some experimental proof of the theoretical models was provided with vesicle systems (artificial lipid bilayer spheres) (64). Since the direct observation of membrane potentials across internal membranes is challenging, the evaluation of subcellular mechanisms has mostly been based on the observation of secondary effects, which can often be explained by the poration of organelle membranes (65). Further complications arise from the complexity of geometries and unknown characteristics of subcellular structures, for example, whether mitochondria are enveloped by a double membrane, and the difference in composition between interior membranes and the outer cell membrane. (The outer membranes of different cell types also show considerable differences in the relative quantities of different membrane proteins.) In addition, the dielectric properties of organelles and other subcellular structures are often unknown. Finally, membranes are not the only constituents that can be affected by nanosecond pulsed electric fields. Molecular structure of the cytoskeleton and genetic molecules are also highly charged and are likely to respond to strong electric fields (66). An example for the response of the cytoskeleton to the exposure of 60-ns pulses is shown in Figure 3. (The cytoskeleton is a network of protein filaments running through the cytoplasm, which provide structure to the cells and anchors for the organelles. It plays an active role in many cellular processes.) In adherent-growing cell lines, a ruffled appearance of the membrane within one minute after the exposure makes apparent the rapid disruption of the cytoskeleton. Within a few minutes, the support structure of the cytoskeleton is gone and cells become round and detach. In addition, exposed cells also showed a reduction in telomere count; these are structures that tether the chromosomes to the nuclear membrane. Similar disruption of cytoskeleton and nuclear membrane was also observed for plant cells (67). Without support structures, cells struggle to survive and become more susceptible to subsequent pulsed electric field exposures. This could also explain why the survival rate is much lower in cells lacking an extensive supporting cytoskeleton such as Jurkat cells. In

these cells, the application of a nanosecond pulsed electric field led to deterioration of the cytoskeleton within seconds of exposure.

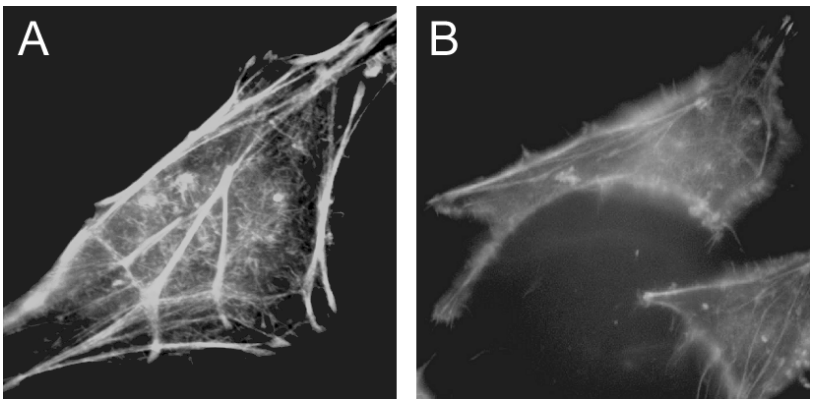

Figure 3. The cytoskeleton of HeLa cells was made visible by staining actin filaments (with Oregon green 488 phalloidin). The cells were growing attached to polylysine coated coverslips. Image A shows the extensive and intact cytoskeleton typical of control cell (sham exposed). Image B shows cells one minute after exposure to a single 60-ns pulsed electric field of 60 kV/cm. The membrane appears ruffled and filaments become less distinct. Four minutes after exposure, the cytoskeleton appears to be dissolved and bright actin spots appear instead. Simultaneously the cell shape becomes spherical and cells lose adhesion to the cover slip.

Nanosecond pulsed electric field exposures could also affect the nucleus and nucleic acids directly (68, 69). Changes in chromatid structures, such as gaps, breaks, and the number of fragments, have been observed for nanosecond pulsed electric field exposures, which are similar to damage that can be induced by ionizing radiation (66, 70). (Chromatids are the two main substructures making up a chromosome. Damage to DNA was assessed using comet assays or standard electrophoresis ladders, which showed much longer comet tails when compared to unexposed cells, indicating DNA fragmentation, particularly for suspension cells. That this damage is most likely caused by the electric field directly and is not a secondary cell response triggered by the exposure is indicated by significant differences in the DNA extracted immediately after exposure. Direct effects on the nucleus have also been found with DNA markers, such as acridine orange (60). The dye is used as a nuclear stain, intercalating with the double strand structure of the DNA. Immediately after the exposure to a nanosecond pulsed electric field, the recorded fluorescence activity decreases. This observation can be interpreted either as a direct effect on the binding sites between DNA molecules and dye, or as an outflow of dye through pores that are forming in the nuclear envelope.

Notwithstanding the significant direct effect on macromolecules and structures, the exchange of molecules and ions across barriers through pores formed by the exposure is still likely to be the most important mechanism to affect cell functions. The concentrations of many of the ions released into the cytosol this way are, under ordinary circumstances, carefully maintained. A sudden change in concentration will lead to a response with the goal of compensating for the imbalance. Since changes in ion concentrations regulate cell functions, nanosecond pulsed electric fields should

likewise provide control of these mechanisms. First proof of this concept was provided in an experiment conducted by Stephen Buescher with neutrophil chemotaxis. When placed in a microreactor between two electrodes, the cells move towards a chemoattractant. The directed movement is temporarily interrupted by the application of a single 300 ns, 45 kV/cm pulse (71).

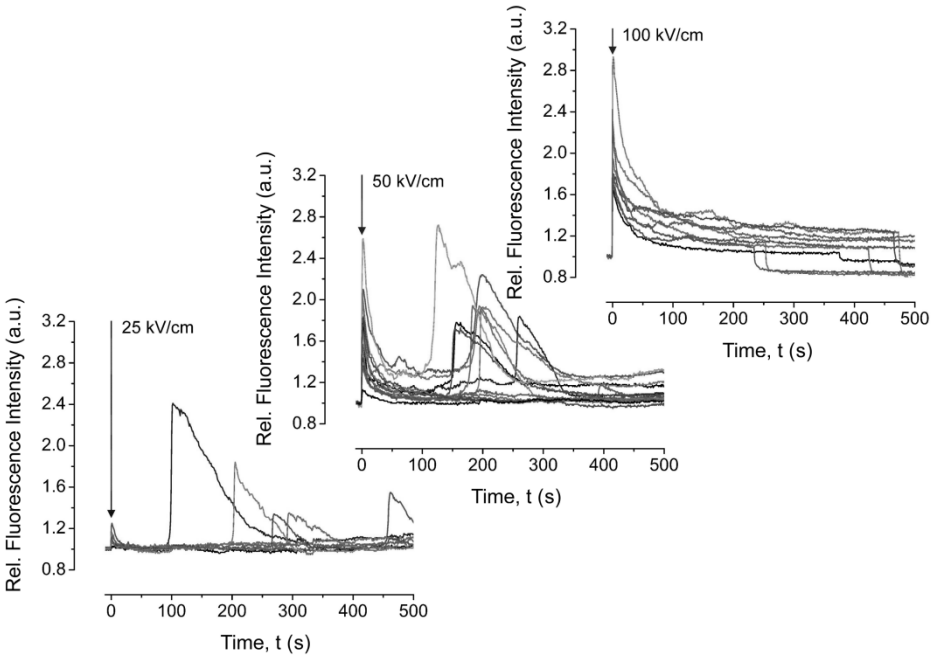

Figure 4. A single pulsed electric fields of 60 ns instigates an immediate, transient release of calcium from intracellular stores (as indicated by the fluorophore fluo-4). The magnitude of the fluorescence response is increasing with field strength. For fields of 50 kV/cm are the induced signals of the same order of magnitude as subsequent natural occurring oscillations. However, for a field of 100 kV/cm are these random changes no longer recurring, indicating sustained subcellular damage. This seems also indicated by the instantaneous drop in fluorescence after several minutes, suggesting loss of membrane integrity.

One of the most important ions involved in intracellular signaling events and chemical signal transmission between cells is calcium. It is stored inside cells in the endoplasmic reticulum and mitochondria and is released in small quantities for signaling events. Cell functions that are mediated by varying calcium concentrations include fertilization, muscle contraction and apoptosis. Calcium responses induced with nanosecond pulsed electric fields have been observed in a variety of cell types with different pulse parameters (71-74). A detailed analysis shows that the release of calcium from internal stores occurs within only a few milliseconds after exposure, again indicating that pores, rather than physiological pathways, are responsible (75). The calcium response is transient and qualitatively similar to physiological signals, as observed for a normal, i.e., unperturbed, cell. This shows that cells deal with the

electrical stimulus in a manner similar to other trigger mechanisms, and calcium is eventually returned to these intracellular stores through calcium pumps. How much calcium is released depends on the pulse parameters (Figure 4). When only moderate increases in concentrations are instigated, calcium activated channels do not seem to activate in the outer cell membrane, and no calcium is taken up from outside. However, if the pulse amplitude is large (with respect to pulse duration and cell type), calcium pathways can incur significant damage. Calcium rushes in through the cell membrane, further increasing calcium concentrations. In this case, cells won't recover from the stimulus; in fact, after several minutes, loss of membrane integrity can be observed, which indicates the death of the cell.

The manipulation of biochemical processes via the regulation of calcium levels offers intriguing possibilities for applications. However, other pathways and messengers might be affected in the same way. Some that have been investigated with respect to triggering of apoptosis are caspases (54, 69, 76, 77).

Applications of Nanosecond Pulsed Electric Field Exposures

As a tool, pulsed electric field exposures offer appealing possibilities for the treatment of cells in medical and environmental applications. The potential to disrupt cell membranes and subcellular components with the goal of killing cells is apparent (19). As such is the method in particular interesting and is investigated for the contamination of liquid foods, e.g. milk and fruit juices, but also for the treatment of waste water and drinking water (20, 25, 78-83). For nanosecond pulsed electric fields, the stimulus can actually reach inside the cell and be used against pathogens that are otherwise protected against agents acting on the cell membrane or chemicals that need to permeate the membrane first (84, 85). The lack of chemical residues is a further advantage for environmental applications.

The unique strength of the method, however, lies in the possibility of instigating more subtle responses (66, 70, 86, 87). Sub-lethal exposure conditions still hold the potential to affect internal structures and membranes. Many of the direct mechanisms are speculative but it seems plausible that proteins, e.g. receptors can be directly affected, for example by breaking individual molecular bonds or by inducing charge shifts along the macromolecules. Subsequently cells will respond with a characteristic signaling cascade and coping mechanism if the stimulus can mimic a familiar stimulus, for example from a chemical compound. Alternatively, protein structures might actually be 'broken' and cells will have to expend repair mechanisms, possibly leading to unforeseen results in the attempt to repair the damage or compensate for it (88, 89). Even the relatively crude process of releasing ions from internal stores, in particular calcium (71, 72, 75), will first of all be interpreted as a biochemical signal. Many of the cell functions that are controlled by intracellular calcium concentrations will respond accordingly (71). This offers in particular a means to control the behavior of specialized cells. These include for example excitable cells and accordingly the effect of nanosecond pulsed electric fields on cell has been shown for cardiac myocytes (90), skeletal muscle cells (91), motoneurons (92), and neurosecretory cells (93).

When applied to platelets, pulsed electric field exposures have been shown to instigate the same response as the enzyme thrombin. The process is also mediated by an increase in intracellular calcium concentrations. As a result platelets aggregate (94). (Platelets are specialized blood cells that are activated to aggregate in wounds and

contribute to coagulation.) Platelet rich plasma is used in surgical and chronic wound care. The activation using the physical (electrical) stimulus avoids complications that have been associated with the use of bovine thrombin and further eliminate the dependency on the protein. Studies that have been conducted to determine the healing rate using platelet gels that have been activated with pulsed electric fields show that wounds heal at least as fast as when the gels are activated with thrombin. In addition, a bactericidal effect of the electric field activated gel was observed, which would help to prevent infections.

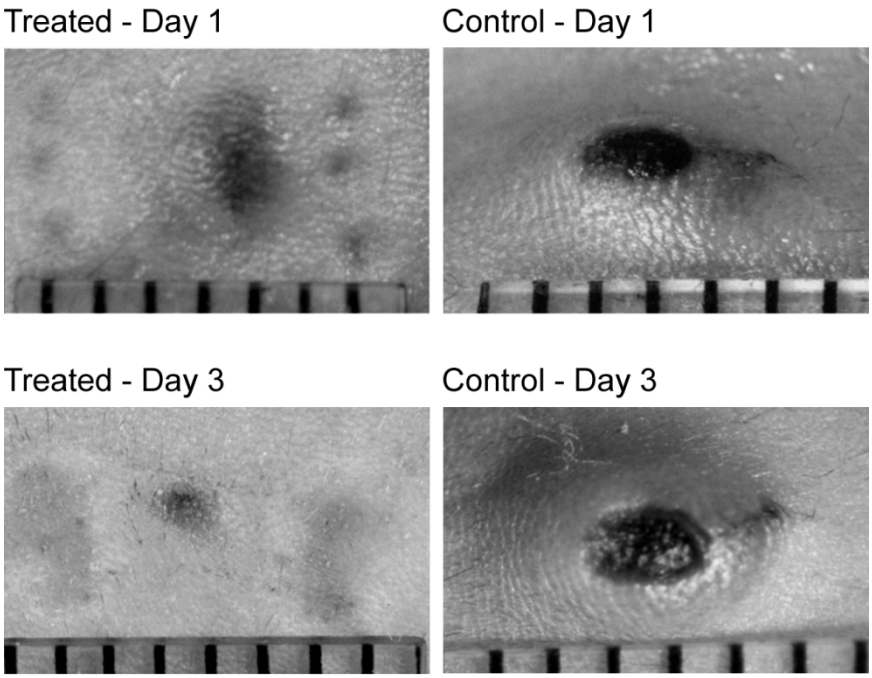

Figure 5. Results of a pilot study comparing untreated melanoma tumors (left column) and melanoma tumors that were treated with a nanosecond pulsed electric field regimen (right column). One million B16f murine melanoma cells were injected into the flanks of C57BL6 mice. When tumors reached a diameter of about 5 mm, nanosecond pulsed electric fields were applied by inserting a pair of needle electrodes (31 gauge hypodermic needles) on either side of the tumor. The electric field was generated by applying a 10-kV high voltage pulse of 300-ns (30 ns rise time) from a Blumlein pulse forming network. For the best possible exposure the electrodes were relocated in 3-4 steps (depending on tumor size) of 1 mm and the treatment repeated at the new location. In each new location 100 pulses were applied. The upper left picture shows the tumor immediately after the first treatment with injection sides clearly visible. The treatment was repeated on the next day. The lower left picture shows the same tumor on the third day. The treated tumor is regressing rapidly, while the control tumor almost doubles in size over the same time.

Perhaps the most interesting application of nanosecond pulsed electric fields involves their capacity to induce apoptosis in cancer cells. (Apoptosis is also known as 'programmed cell death' – a process that is inhibited in cancer cells, leading to uncontrolled cell proliferation.) The interaction mechanisms are still under investigation, but in general, many different processes could be affected by exposure to an electric field (41, 95). Accordingly, many different pathways have been investigated (56, 59, 60, 66, 76, 96, 97). Apoptosis could be instigated by the effect on either the outer membrane or on organelles, such as the mitochondria. Many hallmarks of apoptosis, such as activation of caspases, release of cytochrome c, phosphatidylserene externalization and DNA fragmentation, have been observed and studied with respect to exposure conditions (46, 76, 77, 98-101).

More recently, the efficacy of pulsed electric fields alone as a tumor therapy has been also tested in *in vivo* experiments on different tumor types. Figure 5 shows first results for B16 melanoma tumors grown in mouse skin. By applying 100 pulses of 300 ns duration and 10 kV amplitude in different locations across the tumor a significant reduction in tumor size could be achieved in only one day. No chemotherapeutic drugs were administered in addition. In subsequent studies the treatment conditions could be optimized and eventually a complete remission of tumors was achieved in a group of 17 animals with a treatment regimen applying up to 100 pulses of 300-ns, on different days during a 2 week period (102). (The number of treatments depended on the individual tumor response.) Whereas more of half of the 18 control animals did not survive for more than 3 weeks (and only a few did a little longer), did all of the treated animals live for more than 120 days, demonstrating that they have, in effect, been cured. Tumor cells treated *in vivo* showed many of the same hallmarks of apoptosis that were already observed for cell suspensions and some other features, such as a characteristic nuclear shrinkage (44, 45, 103). In addition, nanosecond pulsed electric fields showed to act not only on individual cells but also had a systemic effect on the tumor by disruption the capability for angiogenesis, hence disruption the tumor's supply with blood (44, 45). In the meantime, similar results and long term survival could also be demonstrated on HEP1-6 liver tumors that were also grown subcutaneously in a mouse model when treated with 100-ns pulses. Many other tumor cell lines have been investigated for their susceptibility at least *in vitro* but some more also *in vivo* (46, 69, 77, 87). In one case a human basal cell carcinoma was successfully treated (46).

Although nanosecond pulsed electric field treatments do not require additional agents, such as chemotherapeutics to be effective, possible synergies might actually enhance a sought-after effect, for example by increasing the permeability of subcellular membranes for chemical compounds (64, 104). The possibility to reach into the cell with an electric field could also offer a way to control cell functions remotely by introducing otherwise inactive substances. A first attempt has been made with carbon nanotubes which were introduced into tumor cells and are expected to respond strongly to an applied electric field due to their unique electrical characteristics (105). Even these newer developments, however, rely on electrode systems that can be brought close to the tumors. Accordingly, targets that can be reached have to be located close to the skin surface or require invasive surgery. A new idea that has emerged, proposes to focus strong electric fields into a patient by using ultrawideband antennas (106). The appealing approach requires shortening the high voltage pulses that have to be applied to the antenna to picoseconds and increase their amplitudes to several tens to hundreds of megavolts per centimeter. Accessing this parameter range poses new challenges and

opportunities for engineering and research. Different physical phenomena and processes will be dominant for these conditions and as a consequence will likely lead to different biological responses as it was already observed for cell viability (107).

Acknowledgment

The insight and knowledge on effects and applications of pulsed electric fields is the result of a dedicated group of researchers on this topic. I was lucky to work with many of them together at the Frank Reidy Research Center for Bioelectrics or to have found them as collaborators at Old Dominion University. Without their ideas and dedication the field would not have advanced as it has. Credit and my thanks for the opportunity to write this summary therefore are extended to E. Stephen Buescher, Peter F. Blackmore, Michael Stacey, Stephen J. Beebe, James R. Swanson, Christopher Osgood, Ravindra P. Joshi, Shu Xiao, M. Arif Malik, Yeong-Jer Chen, Richard Heller, Loree Heller, Olga Pakhomova, Andrei Pakhomov, Barbara Hargrave, Richard Nuccitelli, Angela M. Bowman, Betsy Greogry, W. Hunter Baldwin, Jennifer Pomicter, Wolfgang Frey, Uwe Pliquett, Jue Zhang, Barbara Carroll (also for her help with this and many other manuscritps), Ruth Lyman, many students (too many to list them all) who spend years on the actual experiments and in particular Karl H. Schoenbach for his vision and leadership.

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