2007

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ABSTRACT

Electrical models for biological cells predict that reducing the duration of applied electrical pulses to values below the charging time of the outer cell membrane (which is on the order of 100 ns for mammalian cells) causes a strong increase in the probability of electric field interactions with intracellular structures due to displacement currents. For electric field amplitudes exceeding MV/m, such pulses are also expected to allow access to the cell interior through conduction currents flowing through the permeabilized plasma membrane. In both cases, limiting the duration of the electrical pulses to nanoseconds ensures only nonthermal interactions of the electric field with subcellular structures. This intracellular access allows the manipulation of cell functions. Experimental studies, in which human cells were exposed to pulsed electric fields of up to 30 MV/m amplitude with durations as short as 3 ns, have confirmed this hypothesis and have shown that it is possible to selectively alter the behavior and/or survival of cells. Observed nanosecond pulsed effects at moderate electric fields include intracellular release of calcium and enhanced gene expression, which could have long term implications on cell behavior and function. At increased electric fields, the application of nanosecond pulses induces a type of programmed cell death, apoptosis, in biological cells. Cell survival studies with 10 ns pulses have shown that the viability of the cells scales inversely with the electrical energy density, which is similar to the "dose" effect caused by ionizing radiation. On the other hand, there is experimental evidence that, for pulses of varying durations, the onset of a range of observed biological effects is determined by the electrical charge that is transferred to the cell membrane during pulsing. This leads to a similarity law for nanosecond pulse effects, with the product of electric field intensity, pulse duration, and the square root of the number of pulses as the similarity parameter. The similarity law allows one not only to predict cell viability based on pulse parameters, but has also been shown to be applicable for inducing platelet aggregation, an effect which is triggered by internal calcium release. Applications for nanosecond pulse effects cover a wide range: from a rather simple use as preventing biofouling in cooling water systems, to advanced medical applications, such as gene therapy and tumor treatment. Results of this continuing research are leading to the development of wound healing and skin cancer treatments, which are discussed in some detail.

Index Terms — **Bioelectrics, nanosecond pulsed electric fields, apoptosis, pulse power, subcellular effects, platelet aggregation, tumor treatment, wound healing.**

1 INTRODUCTION

The effect of intense pulsed electric fields on biological cells and tissues has been the topic of research since the late 1950's. Intense means that the electric field is of sufficient magnitude to cause nonlinear changes in cell membranes. The first paper reporting the reversible breakdown of cell membranes when electric fields are applied was published in

1985 [1]. The first report on the increase in permeability of the plasma membrane of a biological cell, an effect subsequently named "electroporation" appeared in 1972 [2]. The electric fields that are required to achieve electroporation depend on the duration of the applied pulse. Typical pulses range from tens of milliseconds with amplitudes of several 100 V/cm to pulses of a few microseconds and several kV/cm.

More recently, the pulse duration range has been shortened into the nanosecond range. The effects of such short pulses have been shown to reach into the cell interior [3]. Pulse durations are as brief as several nanoseconds, with pulse amplitudes as high as 300 kV/cm for short pulses. A new field of research opens, when the pulse duration is decreased even further, into the subnanosecond range. With electric fields reaching values of almost 1 MV/cm, it will be possible to directly affect the structure of macromolecules, such as DNA, in cells and tissue.

This field of research, which, until recently, was a subarea of bioelectromagnetics or bioelectrochemical effects, and recognized by professional societies such the Bioelectromagnetics Society and the Bioelectrochemistry Society, has found acceptance in the pulse power community and dielectric community under the new name "Bioelectrics". There were, and are, special sessions and workshops on this topic at the Modulator Symposium and the Dielectric and Electrical Insulation conference.

This review will provide a brief overview of the state of the research on the biological effects of intense nanosecond pulses. Information on electroporation, where orders of magnitude longer pulses are used, can be found in references [4,5,6,7].

2 MODELING OF THE INTERACTION OF PULSED ELECTRIC FIELDS WITH CELLS

Modeling of pulsed electric field effects on the charge distribution in biological cells ranges from simple analytical models to those generated using molecular dynamics. Molecular dynamics simulations provide the most basic and fundamental approaches for modeling the effect of electric fields on cells. The part of the cell that is to be modeled is considered as a collection of interacting particles. In the case of a cell membrane, which is a lipid bilayer, these particles are DPPC (Dioleoyl-phosphatidyl-choline) molecules that are characterized by charged subgroups. Thus, the structural details on the nanoscale can be included into the simulation for maximum accuracy and relevant physics. For each of the molecules the equation of motion (Newton's equation) is solved, with the force on each charge and dipole within the molecular structure generated by the surrounding charges and dipoles. In spite of its simplicity, this modeling method has only recently [8,9] been used to model cellular membranes under electrical stress [8,9,10,11]. The simulation difficulties include the immense number of particles that need to be considered, and also the small time steps, both requiring the use of high-speed computer clusters. This makes molecular dynamics a computationally intensive approach. As an example, Joshi has used 164,000 particles to model a patch of membrane, with time steps on the order of one femtosecond. This means that modeling small parts of a cell and following the membrane changes numerically can only be done for times on the order of 10-20 ns. Therefore, molecular dynamics, at this point in time, is restricted to modeling small parts of a cell and to ultrashort (ns) pulse effects. The importance of this method is in the visualization of membrane effects on this timescale, the inclusion of complex underlying physics, and the determination of critical electrical fields for membrane changes on the nanoscale, e.g. for pore formation.

This information can be used in less computer intensive, but more comprehensive models (i.e., for describing larger systems, such as an entire cell). In these models the cells are represented as micro resistor-capacitor (RC) units, with the resistive parts being time-dependent. The information from the molecular dynamics can then be used to determine the dynamic resistance for such simpler models and will be discussed in more detail in section 2b. They allow us to determine the response of electric fields on the entire cell, including the plasma and subcellular membranes. However, these models still call for extensive numerical efforts when high spatial resolution is required. So, for quick and simple estimates of the pulsed field effects on cells, molecular dynamics can be used to describe the critical electric fields for pore formation. Although such models will not provide complete information for complicated structures, they can provide guidance to the experimentalist in choosing an appropriate electric field-pulse duration range for bioelectric effects.

2.1 AN ANALYTICAL APPROACH

We will initially focus on a simple analytical, passive, and linear model of the cell. By passive and linear, we mean that changes in the properties of the cell structures, such as electroporation, are not considered. The assumption holds, therefore, only for electric field amplitudes below those required for electroporation or nanoporation. However, it provides useful information on the threshold for the onset of nonlinear effects. After introducing the concept of electroeffects that depend on pulse duration, we will discuss advanced models that include changes in cell structures.

Figure 1a shows a cross-section of a mammalian cell, with the only membrane-bound substructure shown being the nucleus. The cytoplasm, which fills much of the cell, contains dissolved proteins, electrolytes and forms of glucose, and is moderately conductive, as are the nucleoplasm and the contents of other organelles. On the other hand, the membranes that surround the cell and subcellular structures have a low conductivity. We can, therefore, think of the cell as a conductor surrounded by a lossy, insulating envelope, and containing substructures with similar properties. Data on the dielectric constants and conductivities of cell membranes and cytoplasm, as well as nuclear membranes and nucleoplasm, have been obtained using dielectric spectroscopy of cells [12, 13]. Typical values for the plasma membrane of mammalian cells, e.g. B- or T-cells, are: relative permittivities on the order of 10, and conductivities of approximately 10^5 S/m. For the

cytoplasm, the relative permittivity is approximately that of water, 80, and the conductivity is typically one-fifth that of seawater, 1 S/m.

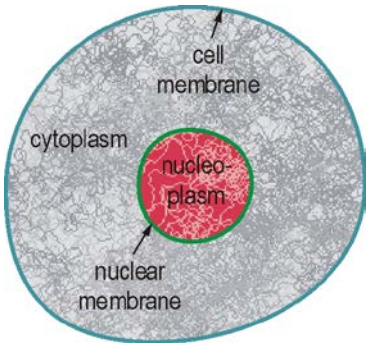


Fig. 1 Cross-section of a cell with nucleus as would be observed with a light microscope. The typical dimension of a mammalian cell (diameter) is on the order of 10 μm.

In the equivalent circuit shown in Figure 2, the conductance of the plasma membrane is assumed to be zero, and the capacitive components of cytoplasm (interior of cell) are neglected. These are assumptions that limit the applicability of the model to a temporal range that is determined by the dielectric relaxation times of membrane and cytoplasm. The dielectric relaxation time, τ_r, provides information on the importance of the resistive or capacitive component of the membrane and cytoplasm, respectively, with respect to the duration of an applied electric field, τ. τ_r is given as:

$$\tau_r = \varepsilon/\sigma \qquad [1]$$

where ε is the permittivity, and σ the conductivity. For a pulse duration, τ, long compared to τ_r, the resistive component dominates, for the opposite case it is the capacitive component.

In order to describe the effect of electrical pulses on cells over a wide range of pulse duration, we need to consider the

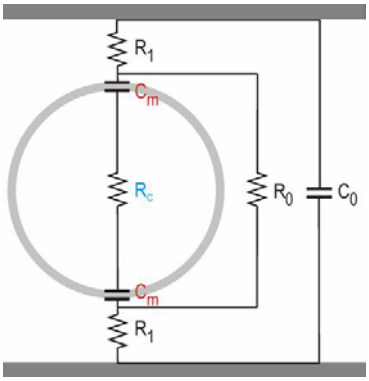


Figure 2 Electrical equivalent circuit of a cell between two electrodes. The cell membrane is described by a capacitance, C_m, the entire cell interior by a resistance, Rc. R_0, R_1, C_0, and C_1, are dependent on the electrical properties of the medium, in which the cell is embedded, and the geometry of the system.

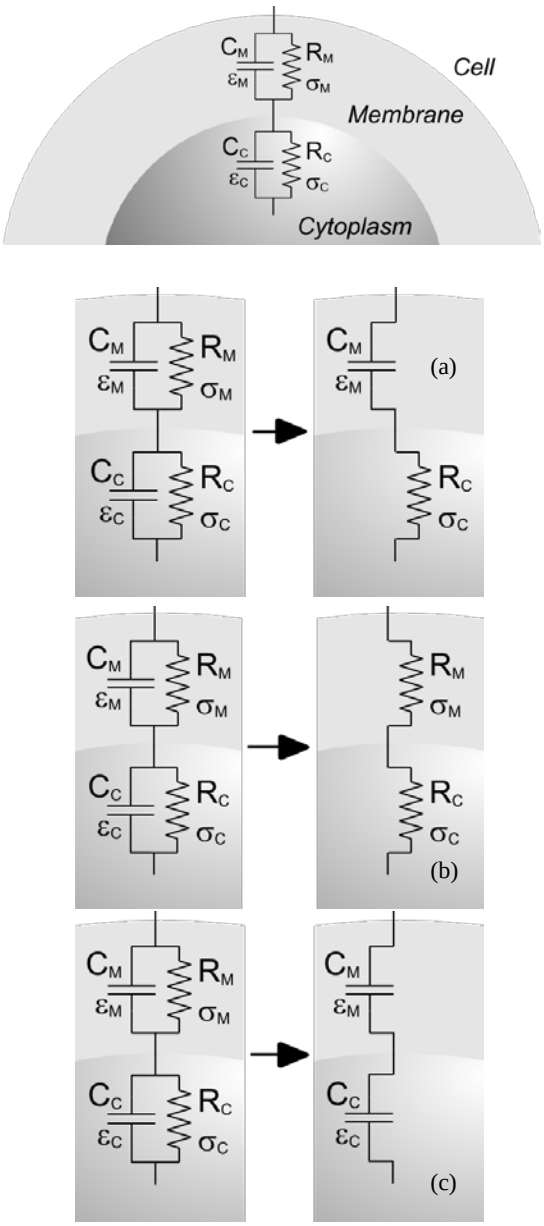


Figure 3 Equivalent circuit of membrane and cytoplasm section of a biological cell. Figure 3a Equivalent circuit used to describe charging of the plasma membrane in the temporal range between the dielectric relaxation time of the membrane and that of the cytoplasm. Figure 3b. Equivalent circuit of a section of a biological cell for long pulses (pulse duration > dielectric relaxation time of the plasma membrane).Figure 3c. Equivalent circuit of a biological cell for extremely short pulses (pulse duration < relaxation time of cytoplasm), generally less than 1 ns for mammalian cells.

equivalent circuit of a cell where these additional circuit elements are taken into account. For simplicity, in the following discussion we will focus on the relevant part of the equivalent circuit only, which includes a section of the plasma membrane and cytoplasm. The equivalent circuit for this case is shown in Figure 3. Although we focus here only on the plasma membrane, it will be shown that the conclusions that are drawn from the discussion of this simple model can easily

be extended to predict electrical effects on the inner cell structures.

The assumptions used in most models for membrane charging and electroporation as well as intracellular electromanipulation are that the membranes are perfect insulators, and that the permittivity of the liquids in and outside the cell can always be neglected. This assumption reduces the general equivalent circuit for a single shell cell to the one shown in Figure 3a. This equivalent circuit is well suited to describe poration processes at cell membranes. It is limited, however, to times that are short relative to the dielectric relaxation time (the ratio of permittivity and conductivity) of the membrane, and to times that are long relative to the dielectric relaxation time of the cytoplasm. Based on data for the electrical properties of mammalian cells [13], these assumptions limit the applicability of this equivalent circuit (Figure 3a) to a pulse duration ranging from approximately one nanosecond to microseconds, depending on cell type.

For this case we can calculate the voltage across the plasma membrane, when a square wave pulse with voltage of $V = E \cdot l$ is applied, where l is the distance between the two electrodes (Figure 2), and E is the electric field in the medium. Starting from the resting voltage, V_r, (which is on the order of 70 mV for many cells), the voltage across the plasma membrane will increase with time until the end of the pulse at time τ, and reach a value of $V_M(\tau)$ +/- V_r at the poles given by [14]:

$$V_M(\tau) = f\, E\, (D/2)\, [1 - \exp(-\tau/\tau_c)] \qquad [2]$$

where f is a geometry factor (1.5 for spherical cells), and D is the diameter of the (spherical) cell. The charging time constant, τ_C, for the cell (plasma) membrane is given as:

$$\tau_c = [\{(1+2V)/(1-V)\}\rho_1/2 + \rho_2]\, C_m a \qquad [3]$$

V is the volume concentration of the spherical cells, ρ_1 is the resistivity of the suspending medium, ρ_2 the resistivity of the cytoplasm, C_m the capacitance of the membrane per unit area, and a is the cell diameter. For a mammalian cell with 10 μm diameter and a volume concentration small compared to one (typical for an in-vitro experiment), the charging time constant is 75 ns.

For long pulses, long meaning that the capacitive term in the membrane impedance can be neglected compared to the resistive term, the equivalent circuit is reduced to two resistors in series. This condition holds for $\varepsilon_M/\sigma_M > \tau$. With the membrane permittivity, ε_M, being approximately $10 \times 8.84\ 10^{-14}$ As/Vcm and the membrane conductivity, σ_M, on the order of 10^{-7} A/Vcm, this condition is satisfied for pulse durations large compared to about 10 μs. Consequently, for pulses of 100 μs duration and longer, capacitive effects can be neglected. The electric field in the membrane at the poles of the cell can be estimated from the condition that the current density at the interface of cytoplasm and membrane is continuous:

$$\sigma_M E_M = \sigma_C E_C \qquad [4]$$

and the condition that the total voltage across a cell, V, is the sum of the voltage across the cytoplasm and twice that of across the membrane. Assuming the electric field along the axis of a cell with diameter D, and membrane thickness, d, is constant in the cytoplasm and in the membrane, respectively, gives us:

$$E_C D + 2E_M d = V. \qquad [5]$$

Combining the two equations allows us to calculate the electric field in the membrane, depending on the applied voltage, V:

$$E_M = V/((\sigma_M/\sigma_C)D + 2d). \qquad [6]$$

Since the conductivity of the cytoplasm is generally on the order of 1 S/m, and the conductivity of the membrane is on the order of 10^{-5} S/m [13], the first term in the denominator is small compared to the second. Consequently:

$$E_M \approx V/2d. \qquad [7]$$

This means that it is reasonable to assume that the entire voltage across a cell in this case is applied across the membranes (at the poles of the cell). This can lead to a strong thermal loading of the cell membranes at these positions. For electroporation to occur, it is generally assumed that the voltage across a membrane needs to reach values on the order of 0.5 to 1 V depending on pulse duration. For an applied electric field of 1 kV/cm, which corresponds to a voltage of 1 V across a cell of 10 μm diameter, the electric field in a membrane of thickness 5 nm is approximately 1 MV/cm. The energy density deposited in the membrane:

$$W = \sigma_M E_M^2 \tau \qquad [8]$$

is then on the order of 10^{-5} J/cm^3 τ. That means that for long pulses, i.e., pulses longer than 100 μs, thermal effects begin to play a role. A 1 ms pulse, e.g. with a voltage of 0.5 V across one membrane would lead to an increase in temperature at the poles (assuming an adiabatic process) of more than 20 degrees.

For very short pulses, the dielectric properties, rather than the resistive characteristics of the media, determine the electric field distribution. The equivalent circuit for a single shell cell is then determined by the dielectric properties only, as shown in Figure 3c. The condition that the resistive term in the cytoplasm impedance can be neglected compared to the capacitive term requires that the pulse duration is short compared to the dielectric relaxation time of the cytoplasm ($\varepsilon_{cp}/\sigma_{cp}$). Assuming that the relative permittivity of the cytoplasm is 80, and the conductivity 1 S/m, this is only true if the pulse duration is less than one nanosecond. The electric fields in the various parts of the cell are then defined by the continuity of the electric flux density:

$$\varepsilon_M E_M = \varepsilon_C E_C \qquad [9]$$

For a membrane with a relative dielectric constant of 8, the electric field in the membrane is ten times higher than the electric field in the adjacent cytoplasm, which has a dielectric constant of 80. For very high applied electric fields of several hundred kV/cm, it might be possible for very short pulses to reach voltage levels across the membrane that cause direct and instant conformational changes of membrane proteins. This range of operation, which is defined by the condition that the pulse duration is shorter than the dielectric relaxation time constant of the cytoplasm, nucleoplasm, and medium surrounding the cells, opens a new temporal domain for cell responses to pulsed electric fields.

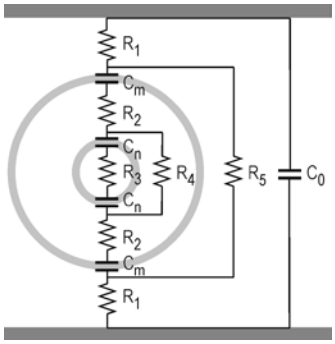


Figure 4 Double shell model of a biological cell, and superimposed equivalent circuit of the cell.

In this analytic approach, substructures of the cell were neglected (the cytoplasm was treated as a homogeneous medium which fills the entire cell. In reality, a mammalian cell contains many substructures: the nucleus, the endoplasmic reticulum, the Golgi apparatus, and mitochondria, to name only a few. Modeling of such cells would require numerical methods as described in the following paragraph. However, as a first step towards a more sophisticated analytical model, it is useful to introduce a two-shell model: a cell that contains only one substructure, e.g. the nucleus. It resembles a cell with a structure as shown in Figure 1. The equivalent circuit of such a cell (which is, for modeling purposes, considered as spherical) is shown in Figure 4. In this case, it is assumed that the plasma membrane and the membrane of the organelle (e.g. the nucleus) are perfect insulators, and that the cytoplasm and the nucleoplasm can be fully described as resistive media. This equivalent circuit provides two time constants, the charging time constant for the plasma membrane and one for the substructure membrane, which is almost always less than that of the plasma membrane.

The charging time constant of the plasma membrane defines the range of intracellular electromanipulation, a research area which has been the focus of multiple, recent studies. The concept of intracellular electromanipulation is easy to understand. During the charging time of the outer membrane, we expect potential differences to be generated across subcellular membranes, an effect which will be stronger, the shorter the pulse rise time is, and can lead to electroporation of subcellular membranes [3]. The subcellular effect will diminish for risetimes long compared to the characteristic charging time of the membranes. Such charging times are in the 100 ns range for human cells, longer for tissue. If direct current electric fields or pulses of long duration (compared to the charging time of the capacitor formed by the outer membrane) are applied, eventually, only the outer membrane will be charged; the electric field generated across subcellular membranes during the charging will be zero for an ideal, fully insulating outer membrane.

2.2 DISTRIBUTED PARAMETER CELL MODELS

The simple analytical approach provides information on the applied electric field intensities required to reach threshold conditions for plasma and subcellular membrane poration dependent on the pulse duration. After the onset of electroporation, this simple linear, passive element approach is no longer applicable to describe electric field-cell interactions. The membrane becomes then an “active” cell element, with variable resistivity and, to a lesser extent, variable permeability. Modeling of cells with “active” membranes has been a topic of publications by the team at Old Dominion University with R. Joshi as principal investigator, and at MIT with Jim Weaver as principal investigator. Briefly, Weaver’s group has used a lattice model [15] and Joshi et al. have used a distributed circuit model with current continuity, and a coupled Smoluchowski equation for pore development [9,16]. These models (as briefly described in the following) are much more sophisticated than the circuit models using fixed values for electrical cell parameters.

A spatially distributed, time-dependent cell model was used by Joshi et al., and included the following features : (i) Discretization of the entire simulation volume into a finite set of grid points that includes the cell, it’s internal sub-organelles, the inner and outer membranes, and a portion of the extra-cellular medium. (ii) An electrical representation of each discrete segment in terms of an effective resistor-capacitor (RC) model. (iii) Application of a coupled nodal analysis at each discretized grid point based on Kirchchoff’s circuit law for the evaluation of time-dependent voltages at each node. (iv) Use of this information to quantify the transmembrane potentials $V_M(t)$ at each requisite membrane. (v) Pore creation by $V_M(t)$ established by the sub-nanosecond voltage pulse. The rate of pore creation depends exponentially on $V_M(t)$. (vi) Characterization of the pores in terms of their spatial and temporal distribution on the basis of the Smoluchowski equation. This includes variations in radii of the pore ensemble. (vii) Dynamical change in pore radii including closure as the external voltage ultimately turned off. Details of the mathematical model and its various aspects are given and discussed next.

In order to facilitate meaningful comparisons with actual experiments, the microscopic electric fields across an actual membrane have to be determined from an experimentally averaged value representing the global voltage applied across the entire sample. Typically, such pulsed experiments are carried out by placing a buffered mixture containing cells into a microcuvette. Thus only the spatially averaged electric field is known from the applied voltage input and physical separation between electrodes. The distributed, nodal analysis accomplishes the task of determining the microscopic voltage

values at both the membrane and the discretized grid points within the cell volume from the global voltage signal.

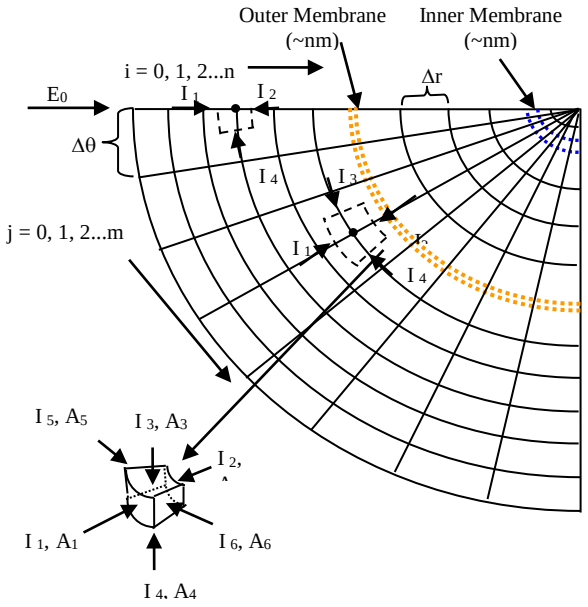


Figure 5 Schematic of the model used to represent a cell for the distributed electrical calculations. The dotted box shows a typical element with current flows.

In this approach, essentially the entire simulation volume is broken up into finite segments, and each segment is represented by a parallel resistor-capacitor (RC) combination to account for the electrical current flow and charging effects as shown typically in Figure 5. The computational region in our simulations is usually taken to be a sector of a sphere that includes the cell, the surrounding suspension medium, and part of the nucleus. In Figure 5, the center of the nucleus is at the r = 0 point, while the intersection of the radial ray with the plasma membrane represents the other boundary. Azimuthal symmetry can be invoked to eliminate the third dimension. The plasma membrane (PM) is denoted as the "outer membrane." The "inner membrane" represents the nuclear membrane. The model also included cytosol between these two membranes, as well as the region within the nucleus. The cell is embedded in an aqueous medium. The outer circular segment defines the overall simulation region boundary. In our simulations, the total radial distance from the center to the PM boundary is chosen according to the typical dimensions of the chosen cell type. Only a full quarter of the cell is typically modelled, and this provides the angular dependence of the transmembrane potential and electric fields, as well as their temporal evolution.

The time-dependent transmembrane voltage $V_M(t)$ produces electropores with a distribution of radii. References to the theory, the resulting equations and their numerical implementation are available in the literature [16,17,18,19, 20,21]. Essentially, the Smoluchowski equation (SE) governs the growth and decay of pores and their evolution in radial-space. The continuum Smoluchowski theory yields the following equation for the pore density distribution function n(r,t), with "r" being the pore radius and "t" the time variable:

$$\partial n(r,t)/\partial t - \{D/(k_B T)\}\partial[n(r,t)(\partial E/\partial r)]/\partial r - D\partial^2 n(r,t)/\partial r^2 + S(r) \quad [10]$$

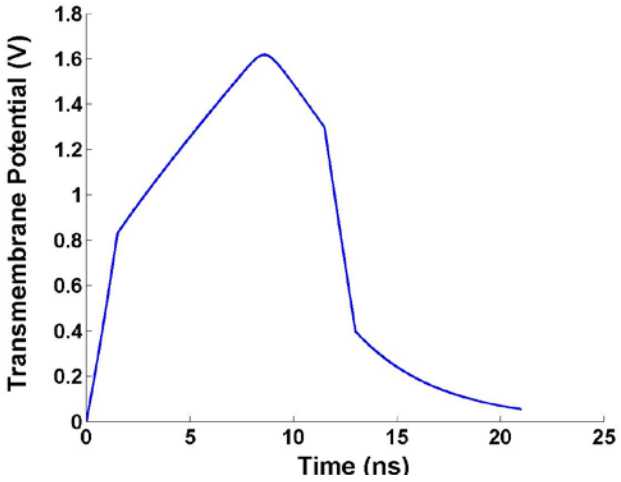
where S(r) is the source (or pore formation) term, while D is a pore diffusion constant, and E(r) is the pore-formation energy. The process of diffusion represents a "random walk" of the pore radius in "r space." Physically, this is brought about by fluctuations in the radius in response to water molecules and other species constantly entering and leaving the pores. The pore formation "S" term depends on the trans-membrane potential and has a strong exponential on its magnitude. Details of the SE, its application and the dynamical aspects of electroporation have been addressed in a series of recent papers [16,22,23] by our group.

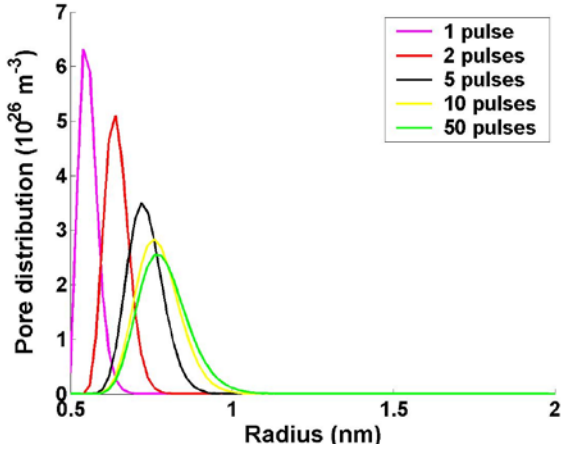
Weaver's group has used a lattice model [24] and the overall approach is similar to the distributed electrical representation discussed here. In the latest Weaver models, triangular mesh generation is used leading to random Voronoi cells within the simulation region of an entire cell. The Smoluchowski equation is also applied as in the present studies for the voltage dependent description of the non-linear membrane resistance. However, the Weaver analyses rely on a simpler asymptotic model for electroporation that uses fixed membrane parameters instead of dynamically evolving values that depend on the instantaneous pore area.

As an example of applying the above distributed circuit approach, the cellular response to a 180 kV/cm trapezoidal pulse with a 10 ns ON time and 1.5 ns rise and fall times is analyzed. The time dependent potential across the plasma membrane at the poles due to this external electrical excitation is shown in Figure 6a. A transient peak of about 1.6 V is predicted during the initial rise time of the pulse. This value slightly exceeds the conventional 1.0 V threshold for membrane poration, and represents a transient overshoot effect. The initial steep rise in transmembrane voltage is driven by the large external displacement current during the 1.5 ns rise time phase. Poration is predicted to occur at times beyond the peak at around 9.2 ns. Following the poration event, the potential is predicted to drop due to the dynamic increase in membrane conductivity. This electrical breakdown of the cellular lipid bilayer represents electro-permeabilization. The sharp drop at about 11.5 ns coincides with the turn-off phase that provides a large negative external displacement current across the plasma membrane. Complete recovery of the membrane voltage is predicted after about 25 ns or so. Values of the transmembrane potential at other angular locations on the membrane (i.e. angle θ varying in the range $0 \leq \theta \leq \pi/2$ radians) were also computed. Though not shown explicitly, their magnitudes at every time instant were smaller than those at the pole (i.e., $\theta = 0$).

Modeling also yields time dependent distributions of the membrane pore population at every discretized angular point on the surface. The highest pore density, as might be expected occur at the pole (i.e., $\theta = 0$) due to the largest electric field present over this region. The generation, growth and dynamic

expansion of pores are included in the current model and it





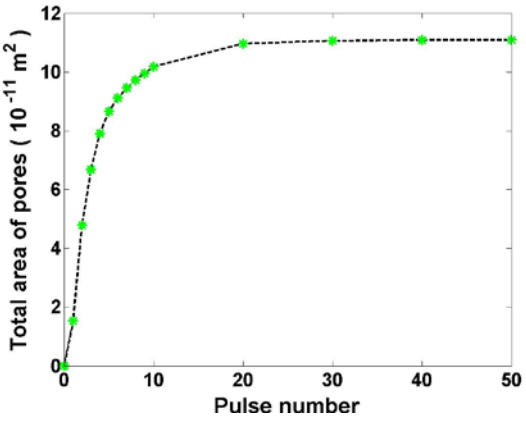


Figure 6a Transmembrane potential across the cell membrane in response to an external 180 kV/cm pulse of duration 10 ns and 1.5 ns rise and fall times.

Figure 6b Pore distribution functions at the pole due to various 10 ns, 180 kV/cm pulse excitations.

Figure 6c Peak total pore area at the plasma membrane for a 10 ns, 180 kV/cm excitation at various pulse numbers.

yields quantitative assessment of the pore distribution. For example, Figure 6b shows the radial distribution in response to 1, 2, 5, 10, and 50 external pulses. The 10 ns, 180 kV/cm pulse with 1.5 ns rise and fall times previously used in connection with Figure 6a was chosen and the simulations carried out for single as well as multiple pulses. As might be expected, Figure 6b shows that the pore distribution shifts to larger values of radius with multiple pulsing. For a train of 50 pulses, for example, the median value of the pore radius is predicted to be about 0.82 nm. For the shortest 1-pulse excitation, a much smaller median value of about 0.55 nm is seen in Figure 6b. In any event, the numerical modeling consistently predicts the pores to have a distribution of radii in the nanometer range. Finally, at longer times and upon higher repetitive pulsing, the pore distribution is seen to broaden due to diffusion in r-space.

The total area of pores created dynamically by the electric pulsing can also be computed at any given membrane by this distributed circuit approach. Such results for the outer plasma membrane of a 12 μm cell, in response to multiple pulsing, are shown in Figure 6c. As might be expected, the total area of pores across the entire surface of the plasma membrane increases monotonically with the number of pulses. A saturating effect is predicted, and is due to the interplay between increasing local conductivity of a porated membrane and the associated trans-membrane potential that drives the growth of pores. With increasing pulses, the pore density is enhanced, leading to larger increases in membrane conductivity. This in turn reduces the trans-membrane potentials. Since pore growth and creation rates are dependent on the transmembrane potential, the reduction effectively leads to progressive decreases in new pore creation. The net effect is the gradual saturation seen in Figure 6c. Physically, any cell membrane can only support a finite amount of poration. This is because the process of poration leads to lipid bilayer molecular shifts and creates local tension that increases dynamically with pore area. Beyond a critical area, collective inter-molecular repulsions begin to emerge and resist any further pore creation and molecular displacement. In the simulations of Figure 6c, the highest area of about 11 x 10^{-11} m^2 roughly represents a peak of 7% poration by surface area. With time, this rapidly reduces as the pores are predicted to reseal upon cessation of the external electric field.

2.3 MOLECULAR DYNAMICS CELL MEMBRANE MODELING

Molecular dynamics, as described in the introductory part of this section, is the most basic approach to understanding electric field-membrane interactions on a nanoscale, both spatial and temporal. Molecular dynamics (MD), as the name implies, is a time-dependent, many-body, kinetic scheme that follows the trajectories of N-interaction bodies subject to chosen external fields. It is a microscopic approach that specifically treats every atom within the chosen simulation region. It relies on the application of classical Newtonian mechanics for the dynamical movement of ions and neutral atoms, taking account of the many-body interactions within a realistic molecular representation of the bio-system. The results of MD calculations shed light on the physics of such interactions, and provide information on critical electric fields for field-membrane interactions. Thus, for example, a segment of the lipid bilayer membrane or a channel protein is first

constructed taking account of the initial geometric arrangement of all the atoms and their bonding angles. Regions of water containing user-specified ion densities are then defined on either side of the membrane to form the total simulation space.

This method is superior to continuum approaches for the following reasons: (i) It allows for the inclusion of collective, many-body interaction potentials at the nanoscale level. (ii) Dynamical screening and dynamical variations in the internal force fields and potentials are automatically included. (iii) It avoids the "mean-field" approximations, (iv) Provides for a natural inclusion of noise and statistical fluctuations. (v) Facilitates self-consistent transport calculations without invoking arbitrary fitting parameters such as the mobility. (vi) Easily facilitates the incorporation of arbitrary defects and non-uniformities, as well as complex geometries.

MD is essential in the present context of high-voltage, ultra-short pulses for the following reasons: (a) The highly non-equilibrium situation precludes the use of "mean-field", "quasi-equilibrium," or "averaged-continuum" theories. (b) Since bio-responses such as conformational changes, poration, etc. are all based on the movement of a small number of actual molecules, a nano-scale atomic-level treatment becomes critical to capture the realistic picture. (c) It also circumvents the need to introduce simulation parameters such as diffusion and rate constants that may not be well characterized, or might have values that differ from their assumed equilibrium levels.

MD simulations were first applied in 1993 to dipalmitoyl-phosphatidylcholine (DPPC) bilayers by Venable et al. [25]. Several studies have since been reported [8,9,10,26,27,28,29,30]. These include simulations of ion-channels, protein conformational changes, and molecular dielectric responses. All of these simulations to date (except for reports by Tieleman [8] and our group [9,10]), have been in the absence of an external electric field. Furthermore, no simulation has been carried out to date that probes the effects of sub-nanosecond, high-voltage pulses.

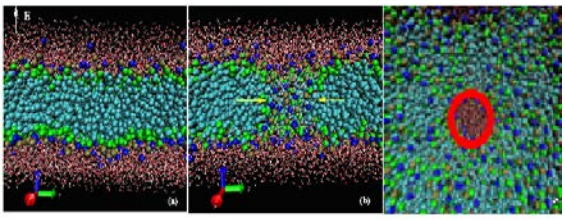


Figure 7 MD results showing nano-pore formation at membrane by an external 0.5 V/nm pulsed electric field.

Results of an MD simulation, shown in Figure 7, for a fixed field of 0.5 V/nm demonstrate nano-pore formation. Choosing a constant electric field was intended to facilitate a rough comparison with recently published data by Tieleman's group [8] where an all-atom model had also been used. Figure 7a shows the initial configuration of the lipid bilayer with aqueous media on either side. Figure 7c shows the top view of the final membrane configuration obtained from MD simulations after about 3.3 ns at the constant field of 0.5 V/nm. The cross-sectional view appears in Figure 7b, and clearly shows the head groups lining the pore. The pore is predicted to be about 1.5 nm in diameter, and somewhat narrower at the central point. This pore formation time was close to the result reported by Tieleman et al. [8]. It may be mentioned that MD correctly predicts the pores to be dynamic with small variations in shape and size, as opposed to the conventional models of rigid cylindrical pores.

3 PULSE GENERATORS FOR BIOELECTRIC STUDIES

The study of intracellular electro-effects and applications of such effects require electrical pulse generators that provide well-defined high voltage pulses with fast current rise to the load. The load could be either cells in suspension or tissue. The applied voltage is determined by the required threshold field for intracellular effects. The value depends strongly on pulse duration: it seems that to achieve comparable effects, the electric field times the pulse duration must be approximately constant. The onset field for any effect is also strongly dependent on the cell type. For single pulses or pulse trains with only a few pulses, generally up to five, typical electric fields for apoptosis range from tens to hundreds of kV/cm. The highest values for 10 ns pulses were 300 kV/cm. These fields are lower for pulse trains with an extended number of pulses.

In order to study intracellular electro-effects, two types of pulse generators have been used. One was developed for experiments which require large numbers of cells, such as those in which flow cytometry is used, or where studies are performed on tissues. The pulse generator is based on the Blumlein concept and can deliver pulses of 10 ns and up to 40 kV into a 10 Ω resistive load. Details are described in a paper by Kolb et al. [B1]. This system serves as a standard pulse source for a consortium of scientists in the Multi-disciplinary University Research Initiative on "Subcellular effects of narrow band and wideband radiofrequency radiation," and is used for experiments or diagnostic methods.

A second class of pulse generators was designed for observations of individual cells under a microscope. Consequently, the gap distance can be reduced to the 100 μm range, and simultaneously, voltage requirements can be relaxed. Instead of typical pulsed power components, low voltage, high frequency cables can be used in the design together with fast semiconductor switches. Such types of pulse generators – micropulsers – operate at voltages of less than one kV. However, because of the small electrode gap, electric field intensities of up to 100 kV/cm are possible and can be applied to cells in suspension that are placed in the electrode gap. This type of pulse generator has been built with variations in pulse length, type of switch, type of pulse forming network, as a line type pulser, and as a hard tube pulser, in many of the ultrashort pulse studies. Circuits and designs are described in references [31,32,33,34]. The compactness of these pulse generators makes them standard tools for ultrashort pulse effect applications.

4 PRIMARY EFFECTS

4.1 EFFECTS ON PLASMA MEMBRANE

The plasma membrane, the outer boundary of the cell, consists of a lipid bilayer with a thickness of approximately 5 nm. The function of the plasma membrane is to protect the cell interior, but it also facilitates the flow of selected types of ions and other materials from and to its surroundings. The plasma membrane has a capacitance of approximately 1 $\mu F/cm^2$, and a resistivity on the order of 10^7 Ωcm [35,36].

The charging of the outer membrane is the predominant primary response of a mammalian cell to an externally applied electric field. With the accumulation of ions along the cell membrane, the potential difference across the membrane increases and the electric field inside the cell is reduced simultaneously. Membrane lipids are perturbed and when a potential difference above a certain threshold is sustained long enough, pores will begin to open and will allow molecular transport. If neither field strength nor exposure time is extensive, the pores will reseal again within several seconds after the electric field has dropped [4,37,38]. The poration effect is related to an increase in membrane conductance, and consequently, a change in transmembrane voltage and current.

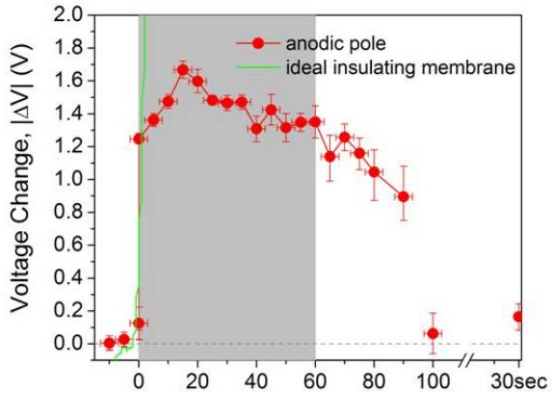


Figure 8 Temporal development of the transmembrane voltage at the anode pole of a Jurkat cell when a 60 ns long 100 kV/cm pulse was applied.

Using laser stroboscopy, we were able to measure the change in transmembrane voltage with a temporal resolution determined by the laser pulse duration of 5 ns [39]. The transmembrane voltage was quantified by staining the cell membrane with a fluorescent voltage sensitive dye. Changes in fluorescence intensity quantify the change in transmembrane voltage. In order to utilize the short illumination time of the laser, the response of the dye needs to be on the same order of magnitude but faster. Recently, a new voltage sensitive dye, Annine-6, was developed with a voltage-regulated fluorescence response that depends only on the shift of energy levels by Stark-effect [40]. This fast electronic process theoretically enables a sub-nanosecond voltage sensitive response.

To investigate the membrane charging in response to an ultrashort pulsed electric field, we stained Jurkat cells (a T-cell leukemia cell line) with Annine-6 and exposed them for 60 ns to an electric field of 100 kV/cm. At different times during the exposure the cells were illuminated with a laser pulse of 5 ns duration (FWHM). The recorded changes in fluorescence intensity allow us to estimate transmembrane voltages based on calibration curves (which were developed for transmembrane voltages of up to 0.3 V, and extrapolated for our results) and monitor their temporal development. The results of measurements at the cathodic pole of a cell with a 60 ns pulse of 100 kV/cm amplitude applied to Jurkat cells are shown in Figure 8.

The results of this study show that an increase in plasma membrane conductivity occurs after only a few nanoseconds (less than the resolution of our method), indicated by the deviation of the measured voltage from the ideal temporal voltage development that would be expected for a passive membrane (green curve). After 20 ns, a second increase in membrane conductance is seen, which might be due to the formation of nanopores [41]. The membrane voltage decrease after the pulse is determined by the discharge of the membrane, but also by the residual applied voltage. The voltage after the 60 ns pulse, does not reach zero immediately.

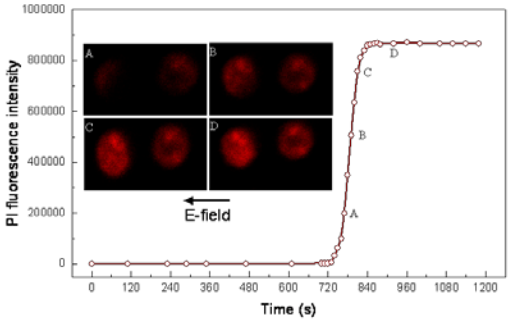


Figure 9 Microscopic real-time image of a typical HL60 cell undergoing PI uptake after (A) 770 s, (B) 790 s, (C) 810 s), and (D) 920 s following a 60 ns, 26 kV/cm electric pulse. The electric field orientation is marked. (reprinted from Biochem. Biophys. Res. Comm. 317:422-427 (2004))

The effects of nanosecond electrical pulses on the plasma membrane are also demonstrated by the uptake of dyes and the orientation of phosphatidylserine in the membrane after applying pulses. Three of dyes most often used to study changes in plasma membrane permeability are trypan blue, propidium iodide (PI), and ethidium homodimer. Figure 9 shows the uptake of PI by HL-60 cells following a single 60 ns, 65 kV/cm pulse [42]. It is interesting that the increase in fluorescence is observed only after approximately 12 minutes and then completed in one minute. This indicates that the formation of pores large enough to allow passage of propidium iodide is a secondary effect, following the formation of nanopores, which occurs on a nanosecond timescale. It is likely that the nanopores are too small to allow PI uptake immediately and the secondary PI uptake is due to membrane failure as *in-vitro*-necrosis, secondary to apoptosis. Usually, uptake of these membrane-integrity dyes is considered an indication of cell death. However, recent experiments with

nanosecond pulses (60ns) [43,44] as well as 0.8ns pulsesindicate that these effects can be transient and are not necessarily harbingers of cell death.

One approach used in these studies was flow cytometry. Here, several thousand cells in suspension flow in a single file through a portal and are individually illuminated by a laser. The scattered light, together with the fluorescent light emitted from these cells provides information on structural or functional features of the cells [45]. When Jurkat cells were exposed to 60ns pulses in the presence of ethidium homodimer and analyzed by flow cytometry, cells exhibited increased fluorescence, indicating that the membrane had been breached to allow uptake of the dye. However, when added 5 minutes after the pulse, no ethidium fluorescence was observed, indicating that the membrane had "resealed" [46]. Likewise, when HCT116 colon carcinoma cells were exposed to three 60ns pulses at 60 kV/cm in the presence of ethidium homodimer, the cells took up the dye up to and even after 1 hour beyond the pulse. However, 5 hours after the pulse, the cells were no longer permeable to the dye. Under these conditions only 10-20% of the cells died, so the reversible effects on the plasma membrane was not due to the elimination of permeable cells. Similar results were observed for Annexin-V-FITC binding, a marker for externalization of phosphatidylserine and apoptosis. Under minimal lethal conditions, these pulses had reversible effects on the plasma membrane integrity and phospholipid orientation. Similar results were observed when B16-F10 cells were exposed to 0.8ns pulses at electric fields between 350-1000 kV/cm with energy densities up to 2kJ/cc using trypan blue. Initially, cells took up the dye. After a certain time, generally about one hour, the membranes of most of the cells seemed to have lost their permeability, and the dye was excluded.

These results indicate that pulsed electric fields have significant effects on the structure of the plasma membrane, but under some conditions the structural changes are reversible. The time required to return to the control state can be considered as a recovery time, presumably to allow "repair" of structure and function. Most importantly, when using pulsed electric fields, these membrane integrity dyes should not be used as cell death markers and Annexin-V-FITC binding may not represent a valid apoptosis marker.

4.2 EFFECT ON SUBCELLULAR MEMBRANES AND ON SUBCELLULAR ORGANELLES

In order to prove the validity of the hypothesis that shorter pulses increasingly affect membranes of subcellular structures, the effect of 60 ns pulses on human eosinophils has been studied [3]. Eosinophils are one of the five different types of white blood cells characterized by large red (i.e., eosinophilic) cytoplasmic granules when the cells are fixed and stained. The cells were loaded with calcein-AM (calcein-acetoxymethylester), an anionic fluorochrome that enters cells freely and becomes trapped in the cytoplasm by an intact cell surface membrane following removal of the AM group. The granules in the eosinophils stay unlabeled because the cytosolic calcein is impermeant to the granular membrane.

When ultrashort pulses with an electric field amplitude of 50 kV/cm and higher were applied to the eosinophils suspended in Hanks Balanced Salt Solution, the granules, which were dark (nonfluorescent) before pulsing, began to fluoresce brightly (Figure 8). This is strong evidence for the breaching of the granule membranes and ionic binding of free calcein from the cytosol to the cationic granule components. On the other hand, the retention of the cytoplasmic calcein staining indicated that the surface (outer membrane) was still intact after the pulsing. Such experiments with 60 ns pulses showed, for the first time, that selective poration/disruption of intracellular membranes can be achieved with ultrashort electrical pulses without loss of the surface membrane integrity [3]. Experiments with shorter pulses (10 ns) have confirmed the earlier results [47].

Similar experimental results have been reported by Tekle et al. [48]. A mixed population of phospholipid vesicles and single COS-7 cells, in which vacuoles were induced by stimulated endocytosis, were exposed to nanosecond pulses. Under appropriate conditions, preferential permeabilization of one vesicle population in a mixed preparation of vesicles of similar size was obtained. It was also shown that vacuoles in COS-7 cells could be selectively permeabilized with little effect on the integrity of the plasma membrane.

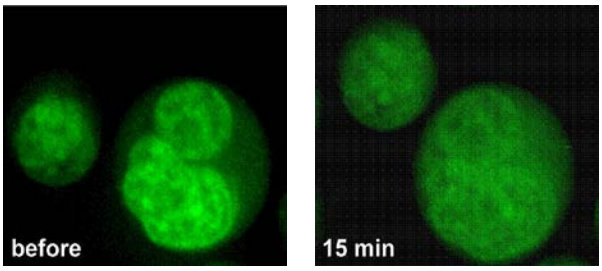


Figure 10 Images of HL-60 cells before and 15 min. after applying a 10ns, 60 kV/cm pulse. The cells were stained with acridine orange (AO) a stain that indicates DNA damage.

The effect of short electrical pulses on the cell nucleus has been explored using acridine orange (AO), a vital fluorescent dye [42]. It is able to permeate the plasma membrane, nuclear and other organelle membranes of living cells, and interacts with DNA and RNA by intercalation or electrostatic attraction, respectively. DNA intercalated AO fluoresces green (488 nm) in the nucleus or mitochondria; RNA, electrostatically bound to AO, fluoresces red (>630 nm). Experiments with HL-60 cells, where 10 ns pulses of 65 kV/cm field amplitude were applied, showed an exponential decrease in average fluorescence intensity in the nucleus with a time constant of approximately 3 minutes. Images of the cell before and after pulses with a 10 ns, 65 kV/cm pulse were applied show that the decrease in fluorescence in the nucleus correlates to an increase in fluorescence in the entire cytoplasm [Figure 10]. These results clearly show that nanosecond pulses cause an increase in the permeability of the nuclear membrane, with a subsequent outflow of labeled DNA.

5 SECONDARY EFFECTS

Whereas the studies described in the previous sections dealt with primary effects, particularly the effect of ultrashort pulses on transport through membranes, in the following we will focus on changes in cell functions in response to ultrashort pulses. Dependent on pulse duration, pulse amplitude, and on the number of pulses in a pulse train, various effects have been observed. For high electric fields, which for 10 ns pulses may need to be higher than 200 kV/cm, lower for longer pulses or pulse trains, apoptosis has been observed [43,45,49,50]. When the pulse amplitude was lowered, calcium release from intracellular stores and subsequent calcium influx through store-operated channels in the plasma membrane was observed [47,49,51,52].

5.1 INTRACELLULAR CALCIUM RELEASE

Ultrashort pulses with electric field amplitudes less than required to induce apoptosis in cells, have been found to trigger physiological responses in cells. Most of the research in the lower range of pulse amplitudes has focused on its effect on the release of intracellular free calcium. Intracellular calcium is stored in endoplasmic reticulum (ER) compartments and mitochondria (and for platelets in α-granules), and calcium release from mitochondria is considered an initiation event of apoptosis [50,51].

When 300 ns long pulses were applied to polymorphonuclear leukocytes, immediate, but transient, rises in the intracellular calcium concentration could be obtained with pulse amplitudes as low as 12 kV/cm [47]. In experiments where the cells were actively crawling over a slide surface, with associated fluctuations in $[Ca^{2+}]$ prior to pulse application (observed by using Fluo-3, a calcium indicator), pulsing caused an abrupt loss of mobility that correlated to the rise in intracellular calcium. The immobilization phase of the cells was found to be dependent on the amplitude of the field. Lowering the electric field allowed the cells to recover more quickly, an effect which has previously been observed when aquatic organisms, such as brine shrimp [52] or hydrozoans [53] were subjected to pulsed electric fields.

In experiments with HL-60 cells, which were loaded with Fura-2, a calcium indicator, and exposed to pulses with 60 ns durations and electric fields that were below those used to induce apoptosis, pulse-induced calcium release was studied [46]. To compare the pulse effect to chemically-induced calcium release, the purinergic agonist UTP was used, which is known to release calcium from the ER. In the absence of extracellular calcium, both UTP and a single 60 ns pulse with 10kV/cm amplitude induced calcium release from intracellular stores. The kinetics of the responses were similar, both were rapid and transient. When calcium was subsequently added to the extracellular medium, an influx of calcium was observed in response to both agonists with similar kinetics. Thus, ultrashort pulses and UTP cause intracellular calcium release followed by influx through calcium channels in the plasma membrane with similar kinetics. The results of this experiment, which indicated that for nanosecond pulses the ER is the initial source of calcium, were confirmed in another experimental approach in HL-60 cells pretreated with thapsigargin, which depletes ER calcium by inhibiting a pump that returns calcium to the ER [54]. Thapsigargin-treated cells released less calcium in response to the ligand UTP and non-ligand nsPEFs. Thus we concluded that nsPEFs mimicked the purinergic agonist and targeted the ER, but it was not clear that the mechanisms were the same [54].

Fluorescence microscopy with temporal resolution of milliseconds confirmed the ER as likely source of calcium following pulse application. Other internal calcium stores, in particular mitochondria, might be affected in a similar way by nanosecond pulses. However, the much smaller size of these organelles in comparison to the volume filled by the ER has prevented the temporal and spatial resolution of the release from these sources so far. The release of calcium was observed within the first 18 ms after exposure to a 60 ns pulse and reaches a maximum in less than a second before it decreases in an exponential manner over 2-3 minutes [55]. (Figure 11) With 25 and 50 kV/cm field strengths, the response is independent of the presence of extracellular calcium. For 100 kV/cm field strength, calcium concentrations increase after the initial peak for another 10 to 25 seconds, which suggests the influx of calcium.

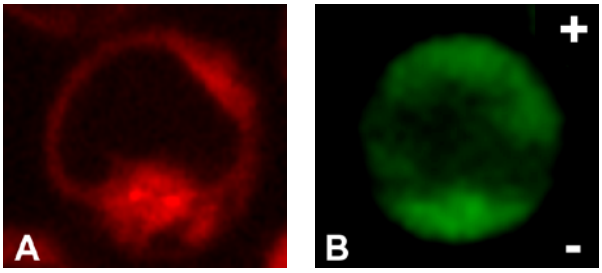


Figure 11 A. A Jurkat cell stained with Bodipy Brefeldin A 558/568, a dye primarily taken up in the endoplasmic reticulum (ER) thus indicating the location of this organelle inside the cell. B. Within 18 ms after the application of a 60 ns pulsed electric field of 100 kV/cm a Jurkat cell shows an increase in intracellular calcium, here indicated by the fluorescent marker Fluo-4 AM. (Image was taken with a camera shutter time of 5 ms.) The comparison of both images shows that calcium is first released from the area of the endoplasmic reticulum and subsequently increases over the entire cell volume as seen with increasing fluorescence intensity.

Nanosecond pulsed electric fields also release calcium from human platelets [54]. This was shown by comparing the effect of a natural platelet agonist, thrombin, with that of nanosecond pulsed electric fields. Thrombin works through receptors in the plasma membrane that also result in the release of calcium from the ER in a manner similar to that used by purinergic agonists indicated above. For platelets, nanosecond pulses did not release less intracellular calcium when calcium was previously released by thrombin from the ER. Likewise, previous treatment with pulsed electric fields did not diminish calcium release by thrombin. Platelets were also pre-treated with thapsigargin to deplete ER calcium levels. When the cells were treated with thrombin, there was a diminished release of intracellular calcium indicating the ER was the calcium source for thrombin. In contrast, the nsPEF-

induced calcium release was not attenuated and was similar to the calcium response to thapsigargin. This suggested that the nsPEF calcium release site in platelets was not the ER, but some other storage site. The platelet alpha granules are likely targets for nsPEFs in this case.

Based on what is known about ligand-induced, physiological calcium mobilization and electric field effects on membranes, it is possible that the pulsed field-induced calcium release could occur by at least one of two mechanisms. One possible mechanism is an effect on the plasma membrane that mimics the ligand-induced, physiological calcium mobilization response acting through G-protein coupled receptors or elements down stream of the receptor. These receptors generate IP3, which binds to specific IP3 receptor channels in the ER for calcium release into the cytoplasm. This is followed by capacitative calcium influx through store-operated channels in the plasma membrane (PM) [56]. UTP, a purinergic ligand agonist, and nsPEFs, a non-ligand agonist, achieved the same calcium mobilization responses indicated above [49]. This could occur by a conformational change and an activation of a PM receptor, or an effect on some other step in the pathway that leads to ER IP3 receptor channel activation. However, at this point, there are no experiments to confirm or deny this mechanism. The other possible mechanism for nsPEF-induced calcium release is suggested by our understanding of electric field effects on membranes, especially those with shorter pulses affecting intracellular membranes, which are not readily affected by conventional PM electroporation [41]. Thus, it is possible that electric field effects have direct supra-electroporation effects on the ER, or other intracellular storage sites releasing intracellular calcium that mimic the purinergic ligand-and thrombin-induced responses. Likewise, a longer pulse duration, higher electric fields, and/or more pulses, will have proportionally greater effects on the plasma membrane, which can result in influx of calcium through pores or aqueous channels.

When considering the thapsigargin experiments, another aspect of thapsigargin action sheds some light onto a possible mechanism of nsPEF effects especially in human platelets. While thapsigargin blocks the uptake of calcium into the ER, some calcium leaks out of the ER down its concentration gradient backward through the pump. Thus the blocked channel acts like a pore to allow calcium to flow out. Since the nsPEF internal calcium release was similar to the thapsigargin response, it is possible that calcium leaked out of pores that could have been caused by a supra-electroporation of a calcium storage site other than the ER, such as the alpha granules. This is consistent with modeling results of Gowrishankar et al. [41].

There are several possible roles for calcium released by nanosecond pulsed electric fields. Calcium is known as a ubiquitous second messenger molecule that regulates a number of responses in cell signaling, including enzyme activation, gene transcription, neurotransmitter release, secretion, muscle contraction, and apoptosis, among others. One known role for nsPEFs-induced calcium mobilization is in platelet activation, which is important for hemostasis and wound healing.

5.2 APOPTOSIS

Mammalian cells use programmed cell death (PCD) to efficiently eliminate themselves from a tissue system when they are no longer functional or necessary (for more information on apoptosis, see e.g. reference 57). This process is different from necrosis in that it does not cause inflammation or gross damage to surrounding healthy cells. One of the most detailed forms of PCD is termed apoptosis, and following an apoptosis-inducing stimulus, several signaling pathways are activated in order to bring about death. These pathways include specific enzymes and signaling molecules that cause distinct morphological changes to the dying cell. The physical changes in the cell reflect the degradation that is occurring to its DNA and proteins and allow it to be recognized by other cells as components that are meant to be scavenged by the immune system. Although the cell is being systematically broken down on the inside the plasma membrane integrity is generally maintained until a much later time so that the enzymes responsible for degradation are not released into the surrounding healthy tissue areas thus avoiding gross damage to the area.

During apoptosis the plasma membrane becomes marked for disposal by phosphatidyl serine (PS) molecules on its surface and, *in vivo*, the task of final removal of this apoptotic body is given to macrophages that engulf the dying cell and finish the breakdown. However, when monitoring apoptosis progression in cells in culture, and thus in the absence of macrophages, the loss of membrane integrity is considered a sign of late apoptosis and is termed secondary necrosis. Phosphatidyl serine externalization following the application of ultrashort pulses has extensively been studied by Vernier et al., using pulses as short as 3 ns [48,58,59,60]. Experiments with Jurkat cells loaded with Calcium Green, a calcium indicator, showed that calcium release is coupled to phosphatidylserine externalization [48]. The pulses applied to the cells had a duration of 30 ns and an amplitude of 25 kV/cm. Although phosphatidylserine externalization is a generally accepted apoptosis marker, it does not necessarily, specifically with this type of stimulation, guarantee cell death.

A wide range of stimuli, of either plasma membrane or intracellular origin, can be responsible for the induction of apoptosis. To study apoptosis signal transduction, one may use pharmacological agents, toxins, radiation, or simply the removal of serum from the culture medium to trigger an apoptotic cascade. Recently it was discovered that the application of intense ultrashort (nanosecond duration) pulsed electric fields (nsPEFs) could cause the elimination of solid skin tumors. Upon investigation of the mechanism of killing in cells in culture, it was found that several of the hallmark indicators of apoptosis could be detected in cells shortly after treatment, including caspase activation, cytochrome c release, phosphatidylserine exposure on the extracellular surface of the plasma membrane, DNA degradation, and the eventual uptake of membrane integrity markers. These observations were exciting in that nsPEFs may be developed as a way to treat tumors without the additional requirement of chemotherapeutic agents, with no residual side effects following treatment, and with limited inflammation at the treatment site.

In order to study apoptosis induction through ultrashort pulses, experiments on Jurkat cells and HL-60 cells subjected to 1 to 5 pulses of 10, 60, and 300 ns were performed with electric fields ranging from 300 kV/cm (for 10 ns) to several ten's of kV/cm for long pulses [45]. The suspension containing the cells was placed in a cuvette between two metal electrodes and, after pulse application, the changes in cell parameters and cell functions were measured using flow cytometry. Apoptosis was induced by ultrashort pulses when the electric field amplitude exceeded a threshold value. For 10 ns pulses, almost 300 kV/cm was required to induce apoptosis in Jurkat cells. For 300 kV/cm, increased Annexin-V-FITC fluorescence is seen, which indicates externalization of phosphatidylserine, a marker for apoptosis. Annexin-V-FITC specifically binds to the cell membrane lipid, phosphatidylserine, which is on the inside of the plasma membrane of non-apoptotic cells, and flips to the outside of the plasma membrane when the cell becomes apoptotic. For longer pulses, e.g. 60 ns, phosphatidylserine externalization was observed at electric fields as low as 40 kV/cm. Whereas Jurkat cells showed signs of apoptosis at relatively low electric fields, HCT116 colon carcinoma cells required higher electric fields to see the same effect [43,44].

Ultrashort pulse induced apoptosis markers appear in tens of minutes following treatment. Following the pulse application, cells bind Annexin-V-FITC and, only later, take up ethidium homodimer. Caspases were activated in 5-20 minutes. The Annexin-V-FITC binding occurred rapidly and permanently and 30% of the cells had proceeded to exhibit membrane rupture by 30 minutes, a typical characteristic of *in vitro*, secondary necrosis. This rapid progression of apoptosis is quite different from that obtained with other apoptotic stimuli, such as UV light and toxic chemicals, which require hours for apoptosis markers to appear. However, the kinetics of ultrashort pulse-induced apoptosis depends on the pulse duration. Shorter pulses result in slower apoptosis progression than longer pulses for the same electrical energy density. Cytochrome c release (another apoptosis marker) was also measured for the presence of caspase activation, when ultrashort pulses were applied to Jurkat cells [45], indicating mitochondria-dependent apoptosis mechanisms. Instead of using single pulses or small numbers of subsequent pulses at high electric fields (> 100 kV/cm at 10 ns), apoptosis can also be induced by applying large numbers of pulses at lower electric field intensity. Vernier et al. [47] reported phosphatidylserine translocation and caspase activation in Jurkat cells with 10 ns pulses of 25 kV/cm amplitude. Unlike the Jurkat cells, rat glioma C6 cells, which normally grow attached to a surface, were found to be highly resistant to the same pulses and pulse sequences. In other experiments, where cell survival after ultrashort pulse application was explored, the results also indicated that non-adherent cells seem to be more sensitive to this application than adherent cells [61].

Since the initial discovery that nsPEFs trigger an apoptosis signaling pathway, there have been significant efforts made to determine the exact mechanism of this process. One of the possible reasons for apoptosis induction was assumed to be extensive calcium release, caused by nanosecond pulses [51]. Calcium is known as a ubiquitous second messenger molecule that regulates a number of responses in cell signaling, including apoptosis. However, in experiments on Jurkat and HL-60 cells [46] it was found that chelation of calcium in the extracellular media with EGTA and in the cytoplasm with BAPTA-AM had little or no effect on caspase activation, suggesting that calcium was not required for nsPEF-induced apoptosis.

Another possible mechanism for apoptosis induction with ultrashort electrical pulses was suggested by Weaver et al. [62]. Ultrashort (nanosecond) pulses are known to affect subcellular structures, either through the displacement current flowing during the early part of the pulse, or through the conduction current after the conductance of the plasma membrane is increased through poration. Consequently, the electric field correlated to the current density in the cytoplasm will affect subcellular membranes, e.g. the mitochondrial membranes. It is known that biochemically-induced apoptosis involves the mitochondrial permeability transition pore complex (MPTP) [63] and/or mitochondrial membrane voltage-dependent anion channels [64]. Weaver's hypothesis is that ultrashort pulses change the transmembrane voltage at mitochondrial membrane sites, which leads to an opening of the MPTP, inducing apoptosis.

The production of reactive oxygen species and subsequent DNA damage has also been proposed as a possible effect [65]. The newest evidence shows that it may indeed be possible for nsPEF stimulation to cause damage to DNA or other critical proteins, directly. Studies aimed at determining this have shown by comet assay that significant DNA damage does occur very quickly after treatment (90 seconds post treatment) with nanosecond pulsed electric fields, and the degree of damage may be temperature dependent [66].

5.3 CELL VIABILITY

The viability of human lymphoma cells (U937 and Jurkat cells) after applying 10 ns long pulses has been studied by Pakhomov et al. using trypan blue uptake as the indicator for cell death [67,68]. Trypan blue was added 24 hours after pulsing to ensure that cell recovery, which occurs on a much faster timescale (see previous section), is not an issue. Results obtained at various electric field intensities are shown in Figure 12, versus the number of pulses for a single pulse duration: 10 nanoseconds.

The survival curves such as those shown in Figure 12 closely resemble well-known types of dose responses for bioeffects of ionizing radiation, and can be conveniently described in the same terms. Disregarding the initial shoulder, the surviving cell fraction $N_{(D)}/N_{(sham)}$ can be calculated as:

$$N_{(D)}/N_{(sham)} = \exp(-D/D_0) \qquad [11]$$

where D is the dose, and D_0 is the dose that decreases the survival to 37%. The initial shoulder can be taken into account using the function:

$$N_{(D)}/N_{(sham)} = 1-(1-\exp(-D/D_0))^n \qquad [12]$$

where n is the extrapolated intercept of the exponential portion of the curve with the ordinate axis. The indices D_0 and n can be used for quantitative comparison of nsPEF sensitivity of different cell lines. Their respective values in our study were 108 J/g and 1.5 for Jurkat cells, and 266 J/g and 1.63 for U937.

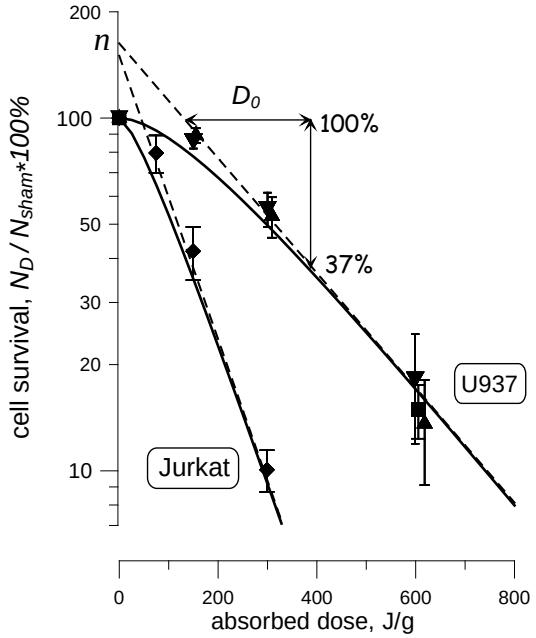


Figure 12 Survival of Jurkat (left) and U937 cells as a function of the absorbed dose. Different symbols for U937 cells correspond to mean survival values (±s.e.) for different pulsed electric fields (87, 125, and 174 kV/cm). Data points at doses producing average survival under 10% have not been included. Dashed and solid lines are the best fits approximations using equations (11) and (12), respectively.

6 A SIMILARITY LAW FOR NANOSECOND PULSE EFFECTS

The results presented in section 5c) clearly show that cell survival or cell viability can be described as a dose effect: the viability is determined by the square of the electric field intensity, E, times the number of pulses, for identical pulse duration, and identical electrical properties of the cell suspension. What is particularly interesting is the weak response to a low number of pulses ("shoulder") in these curves, which is followed by an exponential decline with a further increase in pulse number. This is a well-known, "classical" shape for a survival curve after exposure to ionizing radiation. This is a surprising result, since pulsed electric fields can only be considered nonionizing radiation. It seems that the enormous electric fields used in our experiments are the cause of radicals, setting a similar process in motion as ionizing radiation does. In the ionizing radiation case, reactive species are produced, causing damage of DNA.

The results hold for a given pulse duration: in this case it was 10 ns. Assuming that biological processes caused by ultrashort pulses follow such a dose effect provides us with a similarity law for primary and secondary effects, S_1, of the form:

$$S_1 = S_1(E^2 n) \qquad [13]$$

with n being the number of pulses.

This similarity law holds for a given pulse duration. Varying the pulse duration has been shown to affect the outcome of experimental studies. But rather than showing a scaling with energy, effects observed when the pulse duration was varied scale with the product of electric field intensity times pulse duration [69]. This can be explained by assuming that any observable effect, primary or secondary, is caused by changes in membranes, which requires that a certain critical membrane voltage level be reached. For pulses with duration of less than the characteristic plasma membrane charging time, this critical voltage is a function of the electric charge transported to the membrane. Electric charge, again, can be expressed in terms of the product of applied electric field, E, and the pulse duration, τ, for square wave pulses. This can be shown analytically. According to equ. 2 the electric field required to increase the membrane voltage by ΔV_M is:

$$E = \Delta V_M(2/fD) / [1 - \exp(-\tau/\tau_c)] \qquad [14]$$

where f is a geometry factor, D is the diameter of the (spherical) cell, τ is the pulse duration, and τ_c is the characteristic charging time constant of the membrane. For pulse durations short compared to the charging time constant (which for the plasma membrane of mammalian cells is typically on the order of 100 ns), the exponential function in equ. 14 can be expanded, and equation 10 can be rewritten as:

$$E\tau = \Delta V_M(2\tau_c/fD) \qquad [15]$$

A similar law also holds for subcellular, membrane-bound structures. In this case, this scaling law can be derived from an analytic expression for the voltage increase across a spherical organelle, ΔV_0, in response to an external electric field, E, of duration, τ [14]:

$$\Delta V_0(t) = f^2 E d\tau_c(\exp(-t/\tau_c) - \exp(-t/\tau_0))/2(\tau_c - \tau_0) \qquad [16]$$

where d is the diameter of the organelle, f is a geometry constant (for spherical cells, f = 3/2), and τ_o and τ_c are the charging time constants for organelle membrane and cell membrane, respectively. These time constants depend on size of the cell and organelle, membrane capacitance, and resistivity of medium and cytoplasm. For times short compared to these charging times, the exponential functions can be expanded, with only the first and second term in the expansion being considered:

$$\Delta V_0(t) = f^2 E d\tau_c/2(\tau_c - \tau_o)[1 - t/\tau_c - 1 + t/\tau_o] = (f^2 d/2\tau_o)Et \qquad [17]$$

or

$$E\tau = \Delta V_0(2\tau_0/f^2 d). \quad [18]$$

Accordingly, for times short compared to the charging times of the membranes, the increase in transmembrane potential of organelles is for identical cells dependent on the product of pulse duration and electric field. A second similarity law for nanosecond pulse effect would consequently read:

$$S_2 = S_2(E\tau) \quad [19]$$

Combining the two similarity laws, equs. 13 and 19, into one general similarity law for effects, S, caused by nanosecond pulses is:

$$S = S(E^2 \tau^2 n) \quad \text{or} \quad S = S(E \tau \sqrt{n}) \quad [20]$$

That means that identical effects can be expected when the applied electric field, the pulse duration, and the number of pulses is varied in such a way that the product of $E^2 \tau^2 n$ stays constant. It is based on the hypothesis that primary (and most likely, also secondary) effects are caused by membrane charging. Consequently, the similarity law holds, only if the condition is fulfilled that the electrical field exceeds a value determined by the critical voltage, V_{crit}, for destructive membrane effects:

$$E > E_{crit} = V_{crit}(2/fD) / [1 - \exp(-\tau/\tau_c)] \quad [21]$$

As shown before, for pulse durations short compared to the charging time constant (which for the plasma membrane of mammalian cells is typically on the order of 100 ns), the exponential function in equ. 21 can be expanded, and the equation [21] can be rewritten as:

$$E\tau > (E\tau)_{crit} = V_{crit}(2\tau_c/fD) \quad [22]$$

This condition defines the lower limit in $E\tau$ for which the similarity law (equ. 20) holds. For a critical transmembrane voltage of 1 V across the membrane of a spherical cell with a diameter of 10 μm, and assuming a charging time constant of 100 ns for the plasma membrane, the critical applied electrical field would be 13.3 kV/cm. The upper limit will be determined by saturation processes, such as the fact that the viability can never exceed the value of one, etc.

7 APPLICATIONS

Applications for nanosecond pulse effects cover a wide range: from rather mundane uses, such as biofouling protection for cooling water systems [53], to advanced medical applications, such as gene therapy [46,48] and tumor treatment [70,71]. In this manuscript we have focused on two of them: one, where we are still in research state (wound healing), a second one, where we are on the verge of developing a therapy (tumor treatment).

7.1 PLATELET AGGREGATION AND GROWTH FACTOR RELEASE: A STEP TOWARDS ACCELERATED WOUND HEALING

It has been shown that nanosecond pulses have a similar effect on platelets as thrombin, an agonist that promotes aggregation [72]. Results of studies on the effect are shown in Figure 13. This process of aggregation is initiated by an calcium release from internal stores [54,72]. This is consistent with the well-known fact that aggregation of platelets by known agonists, such as thrombin, requires an increase in intracellular free calcium. The data obtained with nsPEF on calcium mobilization and calcium influx is reminiscent of the well-known capacitative or store-operated calcium entry process induced by hormones (e.g. thrombin) in many non-excitable cells [73]. In this process when intracellular calcium is mobilized from the ER there is an activation of an influx process in the plasma membrane, i.e. the two processes are coupled.

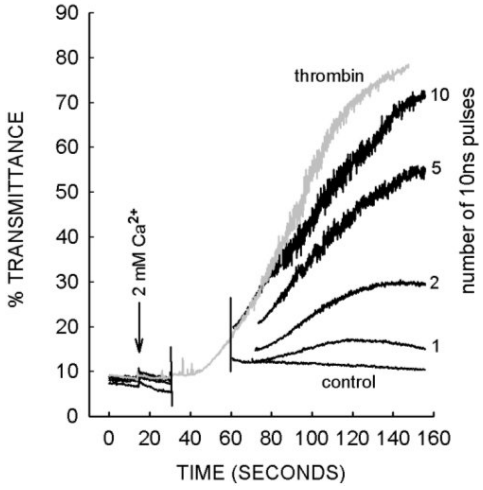


Figure 13 Influence of 10 ns long electrical pulses with electric field amplitudes of 125 kV/cm on the aggregation of platelets. The results obtained with various pulse numbers are compared to the effect of thrombin.

Assuming that the calcium release follows the similarity law [equ. 20], discussed earlier, we should expect that the measured calcium concentration after pulse application for different electric field, pulse duration, and pulse number conditions should be the same for identical values of $E^2\tau^2 n$. Moreover, if the degree of platelet aggregation is correlated to internal calcium mobilization and calcium influx, as has been shown [72], platelet aggregation should also follow the similarity law. This has indeed been shown (Figure 14). Aggregation for 60 ns and 300 ns long pulses at various electric fields and pulse numbers scales linearly with $E\tau\sqrt{n}$. This observed linear increase in aggregation with $E\tau\sqrt{n}$, however, must be only valid as long as we don't approach saturation (100% aggregation).

In addition to aggregation, an increase in PDGF (platelet derived growth factor) released from washed platelets has been observed after pulsing with nanosecond pulses [54,72]. This release is most likely similar due to subcellular electromanipulation, similarly reported in [3]. Platelets are

rich in alpha granule growth factors (i.e. platelet derived growth factor, PDGF, transforming growth factor beta, TGF-β). Release of this growth factor is essential for wound healing.

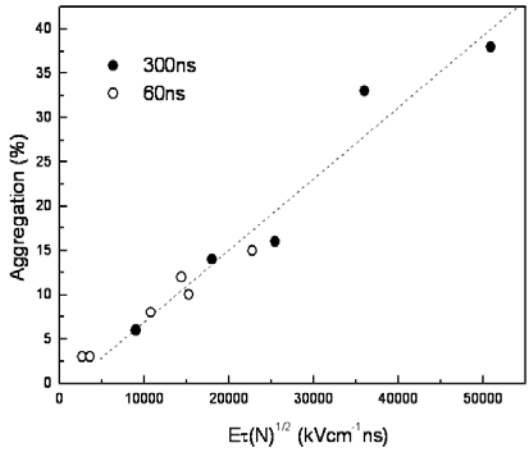


Figure 14 Aggregation of platelets plotted versus the similarity parameter, Eτ√n (electric field intensity times pulse duration time square root of the number of pulses) for pulses with duration of 60 ns and 300 ns.

This concept of using nanosecond pulsed electric fields for wound healing is exciting for several reasons. First, activation of platelets using nsPEF will provide the medical community with an alternative to the use of thrombin (which has been associated with allergic reactions in some patients). Secondly, nsPEF provides a focused, localized stimulus for platelet activation, whereas, other platelet activators such as thrombin, adenosine diphosphate (ADP), thromboxaine or collagen can all enter the circulation and have systemic effects.

7.2 TREATING SKIN CANCER

Electric pulses 300 ns in duration and 40 kV/cm can cause total remission of skin cancer in mice [70]. B16-F10 melanoma cells injected into SKH-1 mice generate melanoma tumors that are 3-5 mm in diameter within 4 days following injection. When these tumors are treated with 300 of the pulses just described, they exhibit three rapid changes. 1) The nuclei of the tumor cells begin to shrink rapidly and the average nuclear area falls by 50% within an hour; 2) Blood flow to the tumor stops within a few minutes and does not return for a week or two; 3) The tumor begins to shrink and within two weeks has shrunk by 90%. Most of the roughly 200 tumors treated begin to grow again at this point, but if treated a second time, will completely disappear. We have begun a long-term study in which we are following animals for 120 days beyond the date when their tumors were no longer visible. Thus far all 13 animals in the treated group exhibit complete remission with no recurrence after 60 days while all 14 control tumors either grew to a size that required that we euthanize the animal or are still present on the animal.

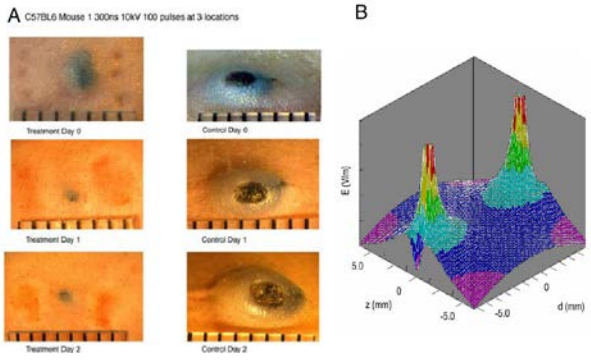


Figure 15 Response of melanoma to 3 applications of 100 pulses using a pair of needle electrodes. A. Treated tumor (left) shrinks following pulse application while control tumor (right) continues to grow. B. Electric field distribution for needle electrode during pulse application.

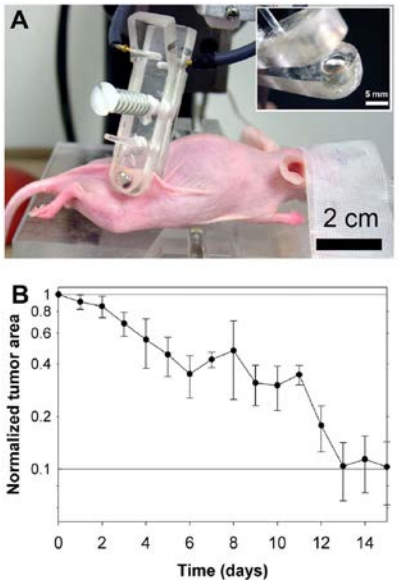


Figure 16 Tumor treatment with parallel plate electrodes. A. Photo of mouse under inhalation anesthesia being treated with clothespin-type parallel plate electrodes. B. Average change in tumor area in three tumors treated with 300 pulses of 300 ns, 40 kV/cm on day 0. (reprinted from Biochem. Biophys. Res. Comm. 343:351-360 (2006))

The pulses were applied with two different electrode types. We started with needle electrodes that penetrated the skin (Figure 15) and then began using parallel plate electrodes in a clothespin configuration (Figure 16a). Parallel plate electrodes have the advantage that all of the tissue placed between the electrodes is exposed to the same electric field, in contrast to needle electrodes, in which the field is much higher near the electrode than far from it (Figure 16b).

These nsPEF stimulate murine melanomas to self-destruct. This is a novel and deadly cellular response to this new nanosecond time domain of pulsed electric field application. While we have not yet tested this technique on humans, it is likely that it may have advantages over the surgical removal of skin lesions because incisions through the dermis often leave scarring on the healed skin. nsPEF kills the tumor without disrupting the dermis so that scarring is less likely. It should also be effective on other tumor types located deeper in the body if a catheter electrode can be guided to the tumor. Among its most intriguing characteristics is the incredibly

short time that these cells have been exposed to the electric field. All of the tumor regression shown here resulted from a total electric field exposure time of 120 μs or less. A second important characteristic is the low energy delivered to the tissue. Each 300 ns pulse of 40 kV/cm delivers only 0.18 J to the tissue between the 5 mm plates. Based on the specific heat capacity of water, this would increase in tissue temperature by only 2-4 ºC. Two seconds between pulses provides more than adequate time for this heat to dissipate so that the tissue will not heat up beyond this. This highly localized and drug-free physical technique offers a promising new therapy for tumor treatment.

8 FROM NANOSECOND TO PICOSECOND PULSES

By reducing the duration of electrical pulses from microseconds into the nanosecond range, the electric field-cell interactions shift increasingly from the plasma (cell) membrane to subcellular structures. Yet another domain of pulsed electric field interactions with cell structures and functions opens when the pulse duration is reduced to values such that membrane charging becomes negligible, and direct electric field-molecular effects determine the biological mechanisms (see section 2c). For mammalian cells, this holds for a pulse duration of less than one nanosecond. In order to study the biological effect of subnanosecond pulses, we have developed a sub-ns pulse generator capable of delivering 250 kV into a high impedance load [77]. The pulse width is approximately 600 ps with a voltage rise of up to 1 MV/ns. The pulses have been applied to B16-F10 (murine melanoma) cells, and the plasma membrane integrity was studied by means of trypan blue exclusion. The results show that temporary nanopores in the plasma membrane are generated, allowing the uptake of drugs or nanoparticles without affecting the viability of the cells.

In addition to entering a new domain of electric field-cell interactions, entering the subnanosecond temporal range will allow us to use near-field-focusing, wideband antennas, rather than needle or plate electrodes, to generate large pulsed electric fields with reasonable spatial resolution in tissue [78]. Modeling results indicate that electric field intensities of tens (up to perhaps hundreds) of kV/cm with a spatial resolution of a few mm can be generated with prolate-spheroidal reflectors with TEM wave-launching structures, and using state-of-the-art pulsed power technology. Moving from the nanosecond to the picosecond range is, in our opinion, the next frontier in bioelectric research.

ACKNOWLEDGEMENT

This work was supported in part by an Air Force Office of Scientific Research/ Department of Defense Multidisciplinary University Research Initiative (MURI) grant on subcellular responses to narrow-band and wide-band radio frequency radiation, administered through Old Dominion University, Norfolk, VA.

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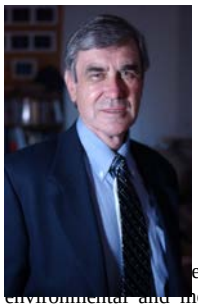

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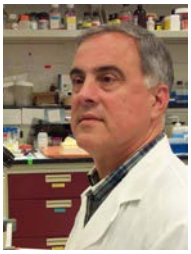

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