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A brief overview of electroporation pulse strength-duration space: A region where additional intracellular effects are expected

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Abstract

Electroporation (EP) of outer cell membranes is widely used in research, biotechnology and medicine. Now intracellular effects by organelle EP are of growing interest, mainly due to nanosecond pulsed electric fields (nsPEF). For perspective, here we provide an approximate overview of EP pulse strength-duration space. This overview locates approximately some known effects and applications in strength-duration space, and includes a region where additional intracellular EP effects are expected. A feature of intracellular EP is direct, electrical redistribution of endogenous biochemicals among cellular compartments. For example, intracellular EP may initiate a multistep process for apoptosis. In this hypothesis, initial EP pulses release calcium from the endoplasmic reticulum, followed by calcium redistribution within the cytoplasm. With further EP pulses calcium penetrates mitochondrial membranes and causes changes that trigger release of cytochrome-c and other death molecules. Apoptosis may therefore occur even in the presence of apoptotic inhibitors, using pulses that are smaller, but longer, than nsPEF.

Keywords

electroporation; cell system; cell model; intracellular electroporation; apoptosis

1. Introduction

Electroporation is now widely accepted as a mechanistic hypothesis relevant to the response of cell membranes to large electric field pulses that rapidly increase the transmembrane voltage, $U_m(t)$, of cell membranes to a value where cell membrane conductance dramatically rises [1, 2]. For essentially the same conditions this leads to molecular transport through cell membranes. In both cases, the changed behavior is often attributed to a burst of pore creation. Recently, attention has expanded to include EP of intra-cellular membranes, with the membranes of progressively smaller organelles experiencing EP as the external electric

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field magnitude is increased. Due to the large size of the the EP literature, we cite only a few papers that reflect existing effects and applications or new, future directions.

An approximate overview of electrical conditions (pulse durations and strengths) for cell and tissue EP-based responses is shown in Fig. 1. Here, “large pulse” indicates that supra-physiologic transmembrane voltages are created at some or all of the sites of a cell membrane. Although early work often focused on artificial planar bilayer membranes, the main motivation now is cell EP, both in vitro (research, biotechnology and industrial processing) and in vivo (clinical research and application). For a typical mammalian cell plasma membrane (PM) this means that U_m rises to at least 200 mV (and in most cases is significantly larger, often reaching 1 to 1.5 V), but the duration is also important (although sometimes invoked, there is no fixed “critical voltage”, because pore creation involves a finite, not infinite, rate).

The pulse strength-duration map (Fig. 1) involves nine orders of magnitude in duration but only three orders of magnitude in strength. This is consistent with the response of cells depending strongly on strength, and relatively weakly on time. This in turn is consistent with the use of an absolute rate equation for pore creation in most electroporation theories employed in spatially distributed cell system models.

The rough guide of Fig. 1 emphasizes some conditions where EP occurs, sufficient to cause established EP effects or applications. Details of the effects and applications are relatively unimportant for this rough guide to electric field pulse strength-duration space. In Section 2 we thus provide only illustrative references and brief descriptions.

There are two rationales for considering strength-duration space. First, the two parameters, “strength” and “duration” of individual pulses are generally chosen by investigators in describing EP protocols. If more than one pulse is used, investigators state how many pulses are used, and report any change in pulse strength and duration during a multiple pulse protocol. Second, “strength” and “duration” are a traditional pair of electrical stimulus parameters. Even irregularly shaped pulses are generally referred to by investigators using the simple “strength” and “duration”, with the implicit understanding that any detailed analysis should consider the actual pulse waveform. However, such complications cannot be part of the simple, approximate map (Fig. 1). For similar reasons, in Section 2 we provide only brief illustrative descriptions and references.”

1.1. Pulse duration

Pulse durations range from approximately 1 nanosecond to almost 1 s (we consider individual pulse widths). Examples are given below in the brief descriptions of EP phenomena and applications.

1.2. Electric field strength

The strength or magnitude of applied electric field pulses range from approximately 100 kV/cm down to 0.1 kV/cm. Because tradition in the EP scientific and engineering literature favors units of V/cm we use these rather than SRI units (V/m). EP pulses of course generate Joule heating, but the pulse duration is small enough that, generally, the temperature rise can be regarded as negligible, at least in terms of causing the main response (membrane pore creation). A temperature rise of a few Kelvin (or °C) does not by itself create significant poration.

2. Effects and applications

2.1. Supra-EP

Exposure to very large fields with durations less than a cell's passive plasma membrane (PM) charging time result in much more numerous, but smaller-sized, pores than conventional electroporation [3] (see below). For the largest fields employed, pores are created in almost all regions of a cell's membranes, including those of small organelles such as mitochondria. One result is that the redistributed electric field mostly goes through cells (isolated or members of a tissue) [4, 5, 6, 7, 8]. This behavior is the opposite to that of conventional EP responses. There is now strong experimental [9, 10, 11]and theoretical [7] support for the interpretation that nsPEF leading to supra-EP involves ionic conduction currents through pores.

However, the distinction between supra- and conventional-EP is somewhat arbitrary: there is no sudden change in behavior as field strength is increased and pulse duration is shortened. Instead, a continuous transition between the two extremes is expected (see "Relatively Unexplored" region of Fig. 1).

2.2. Conventional electroporation

The overwhelming majority of EP investigations and applications involve conventional EP. These involve large pulses in the sense that the transmembrane voltage, U_m, rises to supra-physiologic values, typically at least 0.2 V, but more often near or greater than 1 V for some locations on a cell's PM. Conventional EP involves pulse durations longer than the passive PM charging time (often in the range 0.4 to 1 μs or more). The cell response involves a significant increase in PM conductance at some locations, but for most established conditions, the redistributed field largely passes around the cell [12, 13, 4, 5, 6, 14, 15, 16, 17, 18].

2.3. nsPEF (nanosecond pulsed electric fields)

Since the publication of an attention-getting paper in 2001 that reported intracellular effects due to ultrashort pulses [19], the topic of cellular response to sub-microsecond pulses with mega-volt per meter matnitudes (i.e. 10 kV/cm) has included consideration of 'nanosecond' pulses, even as the pulse duration approaches 1,000 ns (1 μs). A striking feature of cell killing by nsPEF is death by apoptosis [20, 21]. This has motivated significant interest in nsPEF as a basis for treating cancer tumors without delivering drugs or genes [20, 22, 23, 24, 25].

2.4. Electrical injury

Although electrical injury often has a major thermal component that burns tissue, significant and insidious non-thermal damage can also occur. A generally accepted hypothesis is cell death by irreversible EP, leading to necrosis [26, 27, 28, 29, 30, 31].

In the case of ELF (extremely low frequency; widely used 50 – 60 Hz is included) the larger cells (skeletal muscle, nerve) are the most vulnerable. The characteristic "pulse" associated with these periodic fields is taken here to be a half (~10 ms) or quarter cycle (~5 ms). An important insight is that within the human body, large cells (e.g. skeletal muscle, nerve cells) are particularly vulnerable to these relatively long-lasting strong fields, which are in the conventional EP category. The significant differential vulnerability arises from the disparity in cell size, due to membrane-based field amplification (voltage concentration) [32, 33]. In the case of conventional EP, larger cells more readily electroporate than smaller cells for the same magnitude field. Thus electrical shock injury can involve EP-mediated lysis with or

without an elevated temperature from Joule heating, and appears to be consistent with necrotic cell death due to loss of PM integrity.

2.5. IRE (irreversible electroporation)

IRE is closely related to non-thermal electrical shock injury, but is a purposeful, controlled intervention aimed at tumor treatment [34, 35, 36, 37, 38, 39, 40, 41]. These and other studies strongly suggest that IRE is likely to be a highly effective method for tumor ablation. Like all EP-based methods, it does not lose effectiveness at tumor sites near thermally significant blood vessels that provide cooling, and therefore can treat cancer cells that might otherwise survive thermal ablation methods. As in the case of electrical injury, necrotic cell death dominates. Aggressive pursuit of clinical applications is ongoing.

2.6. ECT (electrochemotherapy)

This well established EP-based approach to tumor treatment involves shorter, smaller pulses that typically cause reversible EP, with tumor treatment based on delivery of potent anticancer drugs (usually bleomycin) into cells [42, 43, 44, 45, 46, 47, 48, 49]. Typically, eight square (trapezoidal) pulses of duration 100 μs at one second intervals are used. Without bleomycin present, cells survive, but effective tumor ablation occurs if small (nM) extra-cellular concentrations of bleomycin are provided. ECT is becoming widely used [50], and continues to grow in clinical importance.

2.7. In vivo gene delivery

In vivo delivery of large DNA molecules into cells of viable tissue [51] involves reversible EP created by still smaller and longer pulses (Fig. 1). Often, modification of skeletal muscle or dendritic cells is sought, leading to significantly improved tumor treatment and, perhaps most importantly, enhanced vaccination overall [52, 53, 54, 55, 56]. Relatively small field pulses with longer durations combined with small, long electric field pulses (to electrophoretically mediate transport of highly charged and electrically mobile DNA) and other multiple pulse protocols are commonly employed. This medical and biotechnological application, which relies on cell survival rather than death, is receiving considerable attention and has the prospect of making a major impact.

The delivery of oligonucleotides by EP is also of growing interest [57, 58]. In this case, the highly charged molecules may have a greater probability of being delivered through the PM, with less interception and binding to the membrane prior to stepwise entry into the cell [59].

2.8. Relatively Unexplored region: site of new intracellular effects?

This region in Fig. 1 is somewhat arbitrarily defined by pulse durations from 1 to 100 μs (two of the nine orders of magnitude in pulse duration), but is meant to point to a potentially useful range of pulse strengths and duration yet to be fully investigated. It is located between the declared 1, 000 ns upper limit for nsPEF and the 100 μs lower boundary of most ECT and IRE pulse protocols. However, the designation “Relatively Unexplored” means only that relatively few publications involve these electric field pulses.

Pulses within this region may be well suited to manipulating cells by intracellular-EP, viz. by in situ electroporation of organelle membranes. A recent theory-based cell system modeling paper makes explicit estimates supporting this possibility [18]. Two mechanisms are suggested: (1) Use of sufficiently large (2 to 9 kV/cm) exponentially-decaying pulses with 40 μs time constant to cause EP in progressively smaller organelles (endoplasmic reticulum membrane to nuclear envelope membranes to outer and inner mitochondrial membranes) as pulse strength is increased. (2) Use of a 2 ms trapezoidal pulse (outside the Relatively Unexplored region) that generates sufficient PM poration that intracellular fields

raise transmembrane voltages high enough to gate organelle channels. Thus, some pulses within this region should cause intracellular effects without using nsPEF, suggesting that unusually large conventional EP pulses can be used instead.

2.9. Important prior examples in the "Unexplored Region"

A few examples are given below. In the future there will likely be more, based on more widely ranging research, including both experiments and theory-based cell system models.

Several early experiments (we cite two of eight [60, 61]) with human erythrocytes used pulses with strength and duration that belong to the Relatively Unexplored region of Fig. 1. Since mature (terminally differentiated) human erythrocytes have no organelles [62], intracellular EP would not have been possible. Accordingly, while there were important insights into PM EP there was no hint of intracellular effects.

A first example using cells with organelles is the tremendously influential work that first demonstrated uptake and expression of DNA using mouse cells [63]. This early experiment used "exponential pulses" (exponentially decaying pulses) with time constant 5 μs, applying 3 pulses at 10 min intervals at 20 °C. DNA transfection was observed over the range 6 to 10 kV/cm, with maximum effect at 8 kV/cm. This pulse selection lies in the Relatively Unexplored region. Presently, longer (often millisecond) pulses with smaller strengths are predominantly used for DNA transfection protocols. One reason is that smaller pulses can be more readily generated with simpler pulse generators.

A second example is the use of exponential pulses with a time constant of 40 μs and strengths ranging from 4.5 to 8.1 kV/cm [64]. This study reports several indicators of cell death by apoptosis, yet the electrical conditions (duration and strength) are well outside the nsPEF region. The authors of this important study proposed an explanation based on salt composition and ionic strength of the extracellular medium. As suggested above, an alternative explanation is intracellular EP.

A third important example is "Nucleofection", which uses pulses to deliver DNA directly into the nucleus of eukaryotic cells. Information regarding pulse strength and duration is more readily found in the patent literature than in research publications [65, 66, 67], which typically cite program protocols for pulsing designed by the manufacturer, and are not described in detail. In one general application, pulses are applied in pairs, with an initial large and relatively short pulse (2 – 10 kV/cm, 10 – 200 μs) immediately followed by a smaller but longer pulse of 100 ms maximum duration [68]. Alternatively, a train of 1 to 10 pulses with rest intervals of at least 100 μs is applied, with each pulse having a duration of 10 μs to 5 ms and field strengths varying from 1 to 10 kV/cm [69]. Nucleofection is likely to involve essentially simultaneous EP of the PM and membranes of the nuclear envelope, and regions of the endoplasmic reticulum (ER) membrane are also likely to be electroporated, which could lead to calcium release into the cytoplasm.

2.10. Future Examples

Additional EP phenomena and effects may await discovery. For the stronger pulses in this region, behavior similar to that of supra-EP should be expected. Specifically, more extensive EP is expected to occur, with increased membrane conductance at the very sites that have larger membrane resistance. This means that cell membrane barriers will tend to be greatly decreased, both for the outer (plasma) membrane and progressively for inner (organelle) membranes as pulse field strength is increased.

In view of recent and ongoing improvements in cell system modeling, we argue that a combined computational and experimental approach to research can be rewarding. In silico

cell models can be used to explore pulse parameter space more rapidly than experiments alone. We note two examples. In one, an isolated cell model has demonstrated progressive EP of intracellular (organelle) membranes, with smaller and smaller organelles experiencing EP as pulse strength is increased for an exponential pulse with a 40 μs time constant [18]. In the second example, the same cell system model was used, but a digitized experimental nsPEF pulse waveform [24] was used; this digitized irregular waveform was also applied to an in vivo multicellular model [70].

3. Cell system models and intracellular effects

3.1. General features of cell models

Cell models translate mathematically described field pulses into electrical responses. Formally, these responses include spatially and temporally distributed transmembrane voltages, U_m, in addition to electrical currents within the aqueous electrolytes of a cell model's compartments. Pore creation, pore size evolution, and pore transport properties can also be included. These responses are governed by the time-dependent value of U_m at local membrane sites. However, the dynamic pore populations are also the result of local history, as pores accumulate and eventually vanish differently at membrane sites with different electrical and poration histories.

Solutions to cell system models can therefore be complicated. At any time point, the response is the result of interactions between all of the local models within the cell system model. This means that membrane EP behavior can also be emergent and non-intuitive. The ultimate test, however, is whether the models generate responses that can be tested by comparison with experiments. And this is what is important here: what existing empirical evidence and future experimental results will determine whether intracellular effects occur in this new pulse strength-duration region of interest?

3.2. Models with several different organelles

Although there are a number of valuable cell system models that describe some aspects of cell EP quantitatively, only a few [4, 6, 7, 18, 70] contain several different organelles and the requisite single or double membranes with idealized or irregular geometry along with appropriate resting potential sources. These models were originally motivated by the challenge of understanding cell responses to both nsPEF and conventional EP pulses. However, these same models can be examined for their responses to other pulses (waveforms and strengths). This is a fundamental attribute of cell system models: a response to almost any pulse can be observed.

3.3. Model responses to nsPEF and conventional EP

A broad finding is that while conventional EP pulses almost exclusively alter the PM, the much stronger nsPEF pulses with sub-microsecond durations create many small ion conducting pores early in the pulse [7]. This facilitates massive small ion transport through cell membranes. Once a burst of pore creation occurs, transmembrane currents shift from displacement currents to conduction currents. This breach of the PM means that large intracellular fields can be maintained for a long time. Thus, large fields with durations longer than 1 μs are candidates for causing intracellular effects by organelle EP.

Instead of moving small ions to charge a local membrane area, small ions now move through the membrane by entering and exiting the numerous small pores (conduction current). These large ionic conduction currents pervade the cell, creating large intracellular fields. In the case of nsPEF, as pulse magnitudes increase, progressively smaller organelles

are electroporated, and the intracellular electric field becomes progressively more spatially uniform.

3.4. Model used to show intracellular EP beyond nsPEF

A cell system model based on a 2D Cartesian transport lattice has been recently used to quantitatively describe some responses to large conventional EP pulses (durations longer than one microsecond). Construction and solution of this model are described elsewhere [18].

This first analysis [18] used this cell system model with a 40 μs exponential pulse (1 μs rise time) for several different applied field strengths (e.g. 1, 2, 4 and 7 kV/cm). At an applied field strength of 1 kV/cm, only the polar regions of the PM are electroporated, with slight asymmetry (due mostly to the resting potential source, and partially to irregularly located organelles). At 2 kV/cm the electroporated polar region has expanded, and some of the ER is involved, but with different, unconnected local ER membrane areas now porated. At this point, neither the nuclear membranes nor the mitochondrial membranes have significant pores. By 4 kV/cm the electroporated polar region has expanded further: most of the ER and about half of the nuclear membranes is also electroporated. Finally, upon reaching 7 kV/cm EP has become widespread within the organelle membranes of the model, including the mitochondria. The motivation for considering this particular pulse is an experiment that reported apoptosis, but with an explanation based on the composition and concentration (ionic strength) of the media used [64]. We have instead proposed intracellular EP as an explanation.

A second analysis [70] used the same model with a relatively large IRE trapezoidal pulse (duration of 100 μs and 1 μs rise/fall times, and strength: E_{app} = 2.5 kV/cm). The pulse reaches its maximum value in 1 μs, and after a total of 99 μs the field linearly decreases, reaching zero at 100 μs. In this case we examined the model's response at four different times.

At 10 μs the PM has electroporated near the poles, with slight asymmetry. Several extended sites within the ER have already been significantly electroporated, but the nuclear envelope and mitochondria have not. At 20 μs the number of local ER membrane sites with significant pores has increased only slightly, and the other organelles have not been electroporated. At 99 μs there is actually a small decrease in porated sites. This model behavior emerges because after an initial burst of pore creation early in the pulse, U_m falls from a peak value of ~1.2 V to about ~0.5 V, a value at which pore creation is relatively small. Simultaneously, however, pores are decaying with an assumed mean lifetime of 3 ms, and this accounts for a slight (about 3%) loss of pores at 99 μs. Overall, the model's response to this pulse includes some ER electroporation, potentially to a degree sufficient to release significant amounts of calcium from ER stores.

3.5. Inescapable Joule heating

Although EP itself is a non-thermal response, the application of electric fields unavoidably causes some dissipation (Joule heating). Generally, while it is recognized that this companion process is inescapable, a modest temperature rise alone causes insignificant poration. Nevertheless, very large and/or long pulses can create temperature rises which lead to non-specific cumulative thermal damage [71]. The simplest, most conservative estimate is the adiabatic approximation, which simply assumes that no heat transfer occurs on the time scale of interest.

It is reported that vascular block [72] or blood flow cessation [22, 24] occurs for some electroporating pulses. If normal perfusion exists, then the Pennes bioheat equation provides

quantitative guidance for the temperature rise. Often, thermal damage is estimated by computing an indicator quantity. This is basically an absolute rate estimate that is applied, subject to the condition that only temperature excursions above 42 °C are included. Below 42 °C, however, it is assumed that biological repair mechanisms are adequate. With these considerations in mind, we present estimates of the temperature achieved for simple trapezoidal pulses of 1, 3, 10, 30 and 100 μs duration and 1 μs rise/fall times (see Appendix).

3.6. Models are hypotheses

An important simple fact should be kept in mind: Models are hypotheses [73]. These hypotheses may be relatively complicated, but they can be quantitatively tested. Nevertheless, the responses are those of a model, not a real cell. Their value lies in providing insights more readily, and more rapidly, than by use of experiments alone. The primary response of EP is basically biophysical, presently approximated as emerging solely in small lipid bilayer regions of a cell membrane, which with its many proteins is highly complex [74].

3.7. Non-thermal cell death by electric fields

Here we illustrate the potential significance of intracellular EP generally by considering the outstanding problem of understanding the mechanism(s) of cell death by electric fields. Here we focus on a hypothesis involving intracellular EP.

It has long been known that large electric field pulses used for EP can kill cells without heating being the primary cause [30, 75, 34, 76]. In fact, EP is the basis of ongoing approaches to local cancer tumor treatment in which neither drugs nor genes are introduced. Chronologically, the first approach is based on nsPEF, which generally kills cells by apoptosis [20, 77, 24]. The second approach is based on IRE, which generally causes cell death by necrosis [34, 37, 78]. Strikingly, the largest strength fields apparently kill by apoptosis, while the smaller (but still large) fields kill by necrosis.

But how could bigger lethal fields allow cells to have more control over their own demise: apoptosis rather than necrosis? The illustrative hypothesis presented below features a multistep process using two sets of EP pulses. It builds on biochemical processes that regulate the mitochondrial apoptosis pathway (Fig. 2) [79, 80]. What is new in the illustrative hypothesis is the replacement of some biochemical stimuli with membrane EP stimuli.

In this hypothesis, initial EP pulses release calcium from the endoplasmic reticulum, followed by a delayed calcium redistribution within the cytoplasm. Calcium can also enter from the extracellular space by PM EP. With further EP pulses calcium penetrates mitochondrial membranes and causes mitochondrial disruption that releases cytochrome-c and other death molecules such as SMAC/Diablo, EndoG, and AIF. Apoptosis may therefore occur even in the presence of molecular inhibitors, due to nsPEF or pulses in the Relatively Unexplored region that are smaller, but longer, than nsPEF.

Fig. 2 is reproduced from a 2008 review of biochemical and membrane events for cytochrome c release and apoptosis initiation [80]. Another, very comprehensive review of biochemically-induced apoptosis also emphasizes the important role of cell membranes, both the outer membrane (plasma membrane or PM) and inner, organelle membranes [79]. The illustrative intracellular EP hypothesis unavoidably involves the PM, but explicitly also involves EP of the endoplasmic reticulum (ER) membrane, and both the OMM and IMM (outer- and inner mitochondrial membranes). Below we briefly discuss the key EP features of the hypothesis.

3.8. Details of the hypothesis

A hypothetical protocol begins with a first set of one or more pulses causing EP in the ER membrane, and unavoidably also in the PM. Ca^{2+} then enters the cytoplasm from two sources, the stores within the ER and the extracellular medium. The newly arrived Ca^{2+} then redistributes within the cytoplasm. During a pulse Ca^{2+} redistribution occurs by electrodiffusion. Between pulses redistribution is by diffusion. With time, when the cytosolic Ca^{2+} concentration is sufficiently elevated, additional pulses capable of electroporating both the OMM and IMM (outer- and inner mitochondrial membranes) are applied. This second pulse set delivers Ca^{2+} into both the IMS (intermembrane space) and into the mitochondrial cristae.

The first set of pulses need only release Ca^{2+} from the ER (and in part Ca^{2+} from the extracellular space via the PM), so pulse strength need be only moderately large [18]. In this case, the second pulse set uses greater strength pulses to achieve EP in the OMM and IMM of the smaller mitochondria [18]. For very large pulses only one pulse set is needed, as pulses large enough to cause mitochondrial EP will also cause ER EP.

As shown in the lower left of Fig. 2 one key biochemical event is transfer of Ca^{2+} from the ER into into mitochondria. Two pathways are possible: (I) through electropores in the OMM and IMM, and (II) through the PTP (permeability transition pore, a large protein-based structure). The PTP can gate into the open state if the IMM is depolarized about 100 s or longer [81], which may occur by IMM EP [82]. Low molecular weight (mass) solutes also enter the mitochondria interior.

In an additional role, delivered Ca^{2+} can also mobilize cytochrome c from its bound state to cardiolipin at the outer leaflet of the IMM. Remodeling of the matrix and cristae can also occur with small solute entry.

With time an osmotic influx of water swells and distends the IMM. Eventually, with enough time, the swelling leads to OMM rupture. At this time cytochrome c, and other death molecules are released into the cytoplasm; see Fig. 2 lower central region. For many cells this triggers irreversible initiation of apoptosis.

The net result is a two step process: (1) Ca^{2+} delivery from both the ER and extracellular space to, and into, mitochondria, and (2) IMM distension leading to OMM rupture and release of death molecules (cytochrome c, SMAC, others). Thus, the illustrative hypothesis suggests how intracellular EP might lead to additional effects and applications.

3.9. Intracellular EP of organelles smaller than mitochondria

For both nsPEF [3] and smaller, longer pulses [18], relatively large pulses create such a large PM conductance that the intracellular field approaches the external field. This means that experiments on isolated organelles provide some guidance for intracellular EP conditions.

Very early studies of isolated chromaffin granules (from bovine medullary cells; radii ≈ 120 nm, much smaller than lysosomes) indicated that they could be electroporated at a field strength of 20 kV/cm and duration 150 μs (exponential pulse decay time constant) [83]. These granules are significantly smaller than mitochondria. However, the temperature rise must be considered (about 6 °C for the granules). Organelles somewhat larger than these granules should experience EP at smaller intracellular fields, and are therefore candidates.

Accordingly, we should also consider organelles smaller than mitochondria, such as lysosomes [84]). Lysosomes are known to play a role in cell death through lysosomal-

membrane permeabilization [85]. Lysosome size range is broad [84], with radii of 500 nm to 0.6 μm. This suggests that lysosomes are candidates for intracellular EP while avoiding excessive temperatures. As suggested by this explicit, illustrative qualitative hypothesis, intracellular EP is worthy of further exploration.

4. Summary

Following the important 2001 paper [19] reporting experimental results with intracellular effects by megavolt per meter, sub-microsecond pulses (now often simply “nsPEF”), there has been great interest in the nature of these effects. At the same time, there is corresponding interest in quantitative, mechanistic understanding of how these, and similar, effects are caused.

Here we provide an approximate strength-duration map for several well established EP effects and applications. We then discuss the results of two recent models that explicitly treat intracellular effects associated with single and double-membrane organelles, which are purposefully located at irregular locations within a cell and are characterized by membranes of both idealized (traditional) and irregular (more realistic) geometry. We conclude with presentation of an illustrative mechanistic hypothesis for non-thermal cell death by apoptosis that is based on intracellular EP.

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Appendix

The application of electrical pulses leads to thermal dissipation by Joule heating. Multiple pulse protocols with very large or very long pulses may cause significant cumulative thermal damage. Here we estimate the adiabatic temperature increase from single electrical pulses of different field strength and duration.

$$T_f = T_i + \frac{\sigma}{c\rho}|E|^2 \Delta t \quad (1)$$

Here T_i and T_f are the initial and final temperatures in °C, respectively, σ is the electrical conductivity (1.2 S/m for saline; 0.2 S/m for tissue), c is the specific heat of saline (4 kJ/kg K), ρ is the density of saline (1000 kg/m^3), $|E|$ is the magnitude of the peak field strength in V/m, and Δt is pulse duration in s. The thermal properties are obtained from [86].

Estimated temperature achieved in vitro

Typical in vitro laboratory experiments with isolated cells are carried out at room temperature (not well defined; about 23 ± 2 °C). If the simple view of damage onset only at or above 42 °C is presumed, the maximum temperature rise allowable is about 19 °C. This example assumes that a thermostated exposure/pulsing system set to a higher temperature (e.g. 37 °C) is not employed. Based on the adiabatic approximation, a single pulse of different pulse durations causes the in vitro temperature to exceed 42 °C at the following field strengths: 95 kV/cm (1 μs), 48 kV/cm (3 μs), 25 kV/cm (10 μs), 14 kV/cm (30 μs), and 8 kV/cm (100 μs).

Estimated Temperature Achieved in vivo

Following the above in vitro example, for a temperature rise estimate under the in vivo condition we must reduce the allowable temperature achieved to 42 °C (assumes no febrile subjects), which means that a rise of only 5 °C triggers consideration of accumulated thermal damage (which in turn involves the time to cool to a lower temperature). A bigger rise is possible if the treated site is peripheral, e.g. near the surface of a human arm (~27 °C). Again based on the adiabatic approximation, a single pulse of different pulse durations causes the in vivo temperature to exceed 42 °C at the following field strengths: 50 kV/cm (1 μs), 25 kV/cm (3 μs), 13 kV/cm (10 μs), 8 kV/cm (30 μs), and 4 kV/cm (100 μs).

An important caveat is that the cells of an electroporated tissue will experience intracellular fields and ionic currents. For small fields and low frequencies the tissue conductivity is due to ionic currents that flow around cells, within the interstitial space. However, if PM EP occurs then ionic currents also flow through cells of a tissue. This expectation is supported by both experiment [87, 88] and theory.[16, 8] Based on what is known about isolated cell EP, an electroporated tissue's effective electrical conductivity is expected to increase non-linearly with field strength, to depend on pulse duration, and to exhibit memory (hysteresis). For conditions leading to intracellular EP a larger temperature rise is expected, and is likely to be significant.

The approximate threshold for accumulating thermal damage by membranes and tissue at supraphysiologic temperatures is generally accepted to be be 42 °C. Below 42 °C, biological repair mechanisms are assumed to prevent tissue injury [89].

Highlights

- We review electroporation pulse parameter space.
- A relatively unexplored range of pulse durations may be useful for maximizing intracellular effects.
- Cell system modeling can assist in the understanding and development of experimental electroporation applications.

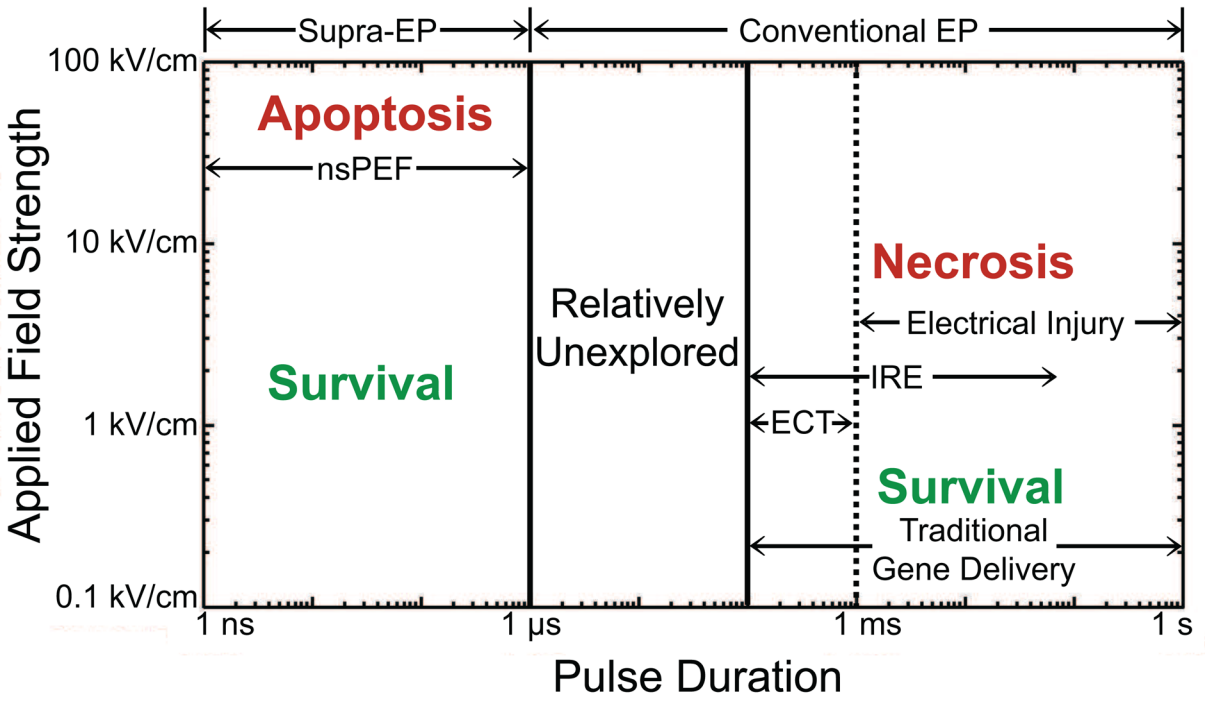

Figure 1. Map of the approximate locations of effects and applications due to cell electroporation (EP) in pulse strength-duration space

The two-dimensional log-log display indicates approximate regions with well established effects and applications. This simple map is therefore a rough guide to much, but certainly not all, EP effects and applications. The vertical position of a label indicates an approximate, associated applied field strength ($|E_{app}|$, magnitude), and the horizontal position of a label indicates a characteristic range of pulse duration. Each phenomenon or application is described briefly in subsections below. The purpose of this figure is to direct attention to a "big picture" of EP, its effects, and applications. Particular attention is directed to the "Relatively Unexplored" region between 1 and 100 μs, where additional intracellular EP effects are expected.

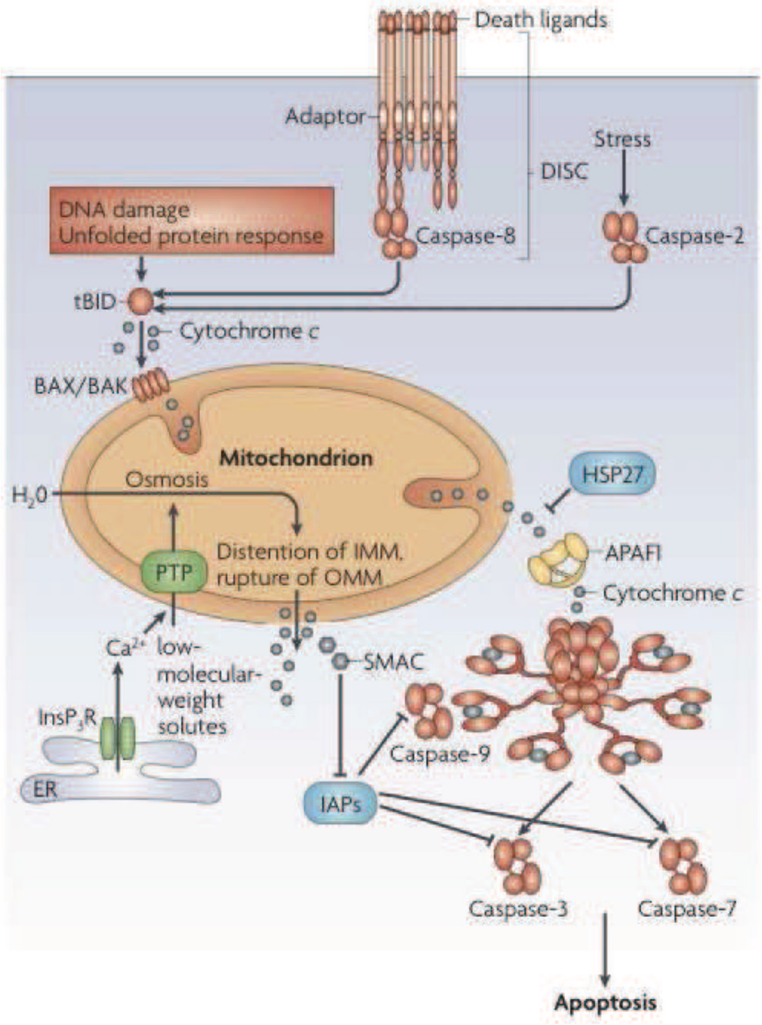

Figure 2. Apoptosis biochemical processes and stimuli [80]

Under normal conditions, pro-apoptotic inner mitochondrial membrane proteins, such as cytochrome c (gray dots) and second mitochondria-derived activator of caspase (SMAC, also known as Diablo; grey hexagons) remain sequestered in the mitochondria. Following an apoptotic stimulus (such as DNA damage, unfolded protein response or stress), BH3 interacting-domain death agonist (BID) is cleaved to yield truncated (t)BID. tBID in turn activates Bcl-2 associated proteins BAX and BAK by inducing their oligomerization to form pores in the outer mitochondrial membrane (OMM). Cytochrome c exits the mitochondrion through these pores, the permeability transition pore (PTP) and/or other channels. Once in the cytosol, cytochrome c binds to apoptotic protease-activating factor-1 (APAF1), enabling its heptamerization and binding to procaspase-9. Activated caspase-9 then activates the executioner caspases-3 and -7. Feedback loops that might promote the release of cytochrome c also exist, such as the opening of the PTP by Ca^{2+} that is released from the endoplasmic reticulum (ER). Opening of the PTP promotes influx of low-molecular-weight solutes into the mitochondria. To counter the proapoptotic events, cytochrome c release can be inhibited by heat-shock protein-27 (HSP27), whereas the caspases can be inhibited by the inhibitors of apoptosis proteins (IAPs). IAPs are inhibited by SMAC. DISC, death-inducing signaling complex; $InsP_3$ R, inositol-1,4,5-triphosphate receptor. Reproduced with permission from Nature Rev. Mol. Cell. Biol. [80].

Table 1

In vitro temperature reached after pulse: Initial temperature is assumed to be 23 °C; electrical conductivity is 1.2 S/m.

Pulse Duration (μs)	Field Strength (kV/cm)				
	1.0	**2.0**	**5.0**	**10**	**30**
1.0	23	23	23	23	26±2 °C
3.0	23	23	23	24	31
10	23	23	24	26	>42
30	23	23	25	32	>42
100	23	24	31	>42	>42

Table 2

In vitro temperature reached after pulse: Initial temperature is assumed to be 37 °C; electrical conductivity is 0.2 S/m (six times smaller than the in vitro case; see caveat below).

Pulse Duration (μs)	Field Strength (kV/cm)				
	1.0	**2.0**	**5.0**	**10**	**30**
1.0	37	37	37	37	37 °C
3.0	37	37	37	37	38
10	37	37	37	38	42
30	37	37	37	39	>42
100	37	37	38	>42	>42