Calcium signaling hacking by MILD electroporation: a novel approach for bioelectronic medicine

Leslie Vallet[1] and Antoni Ivorra[1,2]

[1] Department of Engineering, Universitat Pompeu Fabra, Carrer Roc Boronat 138, 08018, Barcelona, Spain.

[2] Serra Húnter Programme, Universitat Pompeu Fabra, Carrer Roc Boronat 138, 08018, Barcelona, Spain.

Corresponding Author

Address correspondence to:

Leslie Vallet, Department of Engineering, Universitat Pompeu Fabra, Carrer Roc Boronat 138, 08018, Barcelona, Spain. leslie.vallet@upf.edu

Antoni Ivorra, Serra Húnter Programme, Universitat Pompeu Fabra, Carrer Roc Boronat 138, 08018, Barcelona, Spain. antoni.ivorra@upf.edu

Abstract

Electrical stimulation has expanded beyond excitable tissues, with bioelectronic medicine exploring new therapeutic avenues. We propose a novel paradigm: continuously repeated electroporation to induce controlled Ca^{2+} influx and modulate cellular functions. Given the central role of Ca^{2+} as a second messenger tightly regulated by homeostatic mechanisms, transient permeabilization via electric fields enables perturbation of intracellular Ca^{2+} dynamics, influencing processes such as proliferation, differentiation, metabolism and cell death. We define 'MILD electroporation' as a process involving prolonged or repetitive mild membrane permeabilization induced by electric fields that facilitates calcium entry without causing direct cell death. At 5th World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies, we presented *in vitro* evidence showing that specific electric field waveforms elicit Ca^{2+} oscillations in stem cells, modulating gene expression and promoting proliferation. We also presented preliminary results suggesting that burst-modulated alternating fields may slow cancer cell proliferation.

Keywords: electroporation, calcium signaling, permeabilization

Introduction

The use of electrical pulses to generate action potentials for treating neurological conditions, managing cardiac arrhythmias, and implementing neuroprostheses has been a longstanding approach with decades of history. For instance, deep brain stimulation is performed to treat Parkinson's disease, pacemakers are used to manage cardiac arrythmias and cochlear implants are employed to restore hearing. More recently, there has been growing interest in using electrical stimulation, particularly targeting the autonomic nervous system, to treat conditions not traditionally associated with excitable tissues such as rheumatoid arthritis or hypertension[1,2]. A key catalyst for this shift was the publication of the comment article "A Jump-Start for Electroceuticals" in Nature in 2013, which announced that the pharmaceutical giant GlaxoSmithKline (GSK) would lead an academic-industrial initiative that eventually gave rise to Galvani Bioelectronics, a joint venture between GSK and Verily Life Sciences (an Alphabet subsidiary)[3]. This development sparked significant interest, with other pharmaceutical companies beginning to take similar steps[4]. This movement has paved the way for the treatment of conditions traditionally managed with medications using electrical stimulation, thus popularizing terms like "bioelectronic medicines" and "electroceuticals" [5].

However, electrical stimulation of excitable tissues is not the only way to modulate cellular behavior. Here, we advocate for the use of continuously repeated electroporation to artificially trigger the uptake of extracellular calcium ions (Ca^{2+}), thereby altering cellular functions and influencing organ behavior.

Ca^{2+} is an indispensable second messenger in many cell signaling events[6]. Under physiological conditions, its cytosolic concentration is tightly regulated at around 100 nM at rest, whereas the extracellular concentration is approximately 2 mM [6]. Cytosolic Ca^{2+} is regulated by multiple actors working together to maintain homeostasis [7]. If the uptake of extracellular calcium is facilitated by electroporation, it will evidently impact cell signaling and, consequently, cellular processes. This effect will be particularly pronounced when the uptake occurs in repeated bursts rather than as a continuous, low-level influx. Such bursts will overwhelm the homeostatic control mechanisms, which are well equipped for maintaining a stable intracellular calcium level and managing moderate influxes[8], but will struggle to immediately regulate sudden calcium surges.

We hypothesize that by setting the frequency and intensity of calcium uptake triggered by adjusting the applied electric waveforms, various cellular processes can be controlled. For instance, cell proliferation could be halted to inhibit tumor growth, or conversely, promoted to accelerate injury repair. Similarly, this approach could also promote cell differentiation, which could be applied for tissue regeneration and repair. Furthermore, it could also be applied to regulate cell metabolism and stimulate the release of neurotransmitters and hormones, potentially influencing metabolic processes, immune responses, or other physiological functions, opening possibilities for targeted therapeutic interventions [9,10].

The continuously repeated electroporation paradigm

Chronic or semi-chronic delivery of electric currents for therapeutic purposes is well-established. It is applied in proven therapies like cardiac pacing and electrical stimulation for pain management. Additionally, it is utilized in treatments with unclear mechanisms of action, such as bone fracture[11] and cartilage repair [12] and the so-called Tumor Treating Fields (TTFs)[13–16].

In contrast, the idea of continuously repeated electroporation may seem highly unorthodox, as typical electroporation treatments last only seconds or minutes, with total exposure times often measured in fractions of a second. Nevertheless, it is important to recognize that the effects of electroporation continue well beyond the application of the electric field. The cell membrane remains in a permeabilized state for several seconds, minutes or even hours after exposure [17,18]. Therefore, the concept of maintaining a prolonged state of high permeability or applying repeated, intense permeabilization is more aligned with conventional electroporation practices than it may initially appear.

Clinically, the delivery of electric fields to achieve prolonged, intermittent electroporation for calcium uptake could be implemented using approaches similar to those employed in therapies involving chronic or semi-chronic delivery of electric currents. This would involve the use of electrodes energized by portable, wearable, or implanted generators, depending on the specific application. Monophasic pulses would have to be avoided for minimizing electrochemical reactions at the electrode-tissue interface. Similarly, long-duration pulses would need to be excluded to reduce the risk of unintended electrical stimulation[19]. Furthermore, the total applied energy would have to be carefully controlled to prevent excessive Joule heating. Taken together, these considerations suggest that appropriate waveforms for intermittent *in vivo* electroporation may include bursts of short (<10 µs) biphasic pulses.

It is also worth noting that electroporation-mediated Ca^{2+} uptake may already play a significant role in certain therapies that involve the prolonged delivery of electric currents. For example, as discussed later, we hypothesize that electroporation-mediated Ca^{2+} uptake could contribute to TTFs. Additionally, we have already provided evidence suggesting that Ca^{2+} influx induced by electroporation is likely involved in Pulsed Radiofrequency (PRF), a treatment commonly used for pain management [20].

We introduce the term 'MILD electroporation', where MILD stands for Moderate Intensity and Lengthy Delivery, to describe a process characterized by prolonged or repetitive membrane permeabilization induced by field exposures less severe than those commonly applied in conventional electroporation treatments.

Ca^{2+} signaling in a context of Ca^{2+} homeostasis

Despite its seemingly simple nature as a cation, Ca^{2+} is a cellular second messenger of the utmost importance, whose signaling has evolved to be incredibly finely tuned. This owes to the very specific compartmentalization of Ca^{2+} within the cell, where biological membranes confine substantial gradients (e.g., $[Ca^{2+}]_{cytosol} \approx 10^{-7}$ M vs. $[Ca^{2+}]_{extracellular} \approx 10^{-3}$ M, or $[Ca^{2+}]_{endoplasmic\ reticulum} \approx 10^{-4}$-$10^{-3}$ M [21,22]), along with its chemical properties [23] that confer its binding kinetics to proteins [24,25]. From an evolutionary perspective, it has been suggested that the cytosolic Ca^{2+} concentration might reflect the initial Ca^{2+} concentration in the primordial soup [25,26]. While there is no consensus, there is no debate that it was necessary to maintain low cytosolic Ca^{2+} levels (whether the environmental Ca^{2+} concentration increased over time or was always high), as Ca^{2+} at high concentrations tends to precipitate phosphate, involved in the energy cell currency, and DNA/RNA, the carriers of the cell genetic information [6,23,25]. The maintenance of Ca^{2+} homeostasis, *i.e.*, the maintenance of this specific Ca^{2+} compartmentalization within the cell, represents an energy expenditure, but is necessary to avoid cell damage [27] and for cell function, as it is tightly entangled with the Ca^{2+} signaling systems evolved by the cell. Indeed, the same Ca^{2+} channels, pumps and buffers (proteins or organelles that function as Ca^{2+} sinks or stores) maintain Ca^{2+} homeostasis and shape Ca^{2+} signaling [28,29] (Figure 1). Should it

remain spatially and temporally controlled, deviations from the resting 10^{-7} M in the cytosol are fully tolerated and occur as part of physiological Ca^{2+} signaling [23]. As a result, the vast majority of Ca^{2+} signaling systems function by generating Ca^{2+} transients that, beyond avoiding deleterious prolonged elevations of cytosolic Ca^{2+}, allow for finely tuned, graded responses to stimuli [8,30].

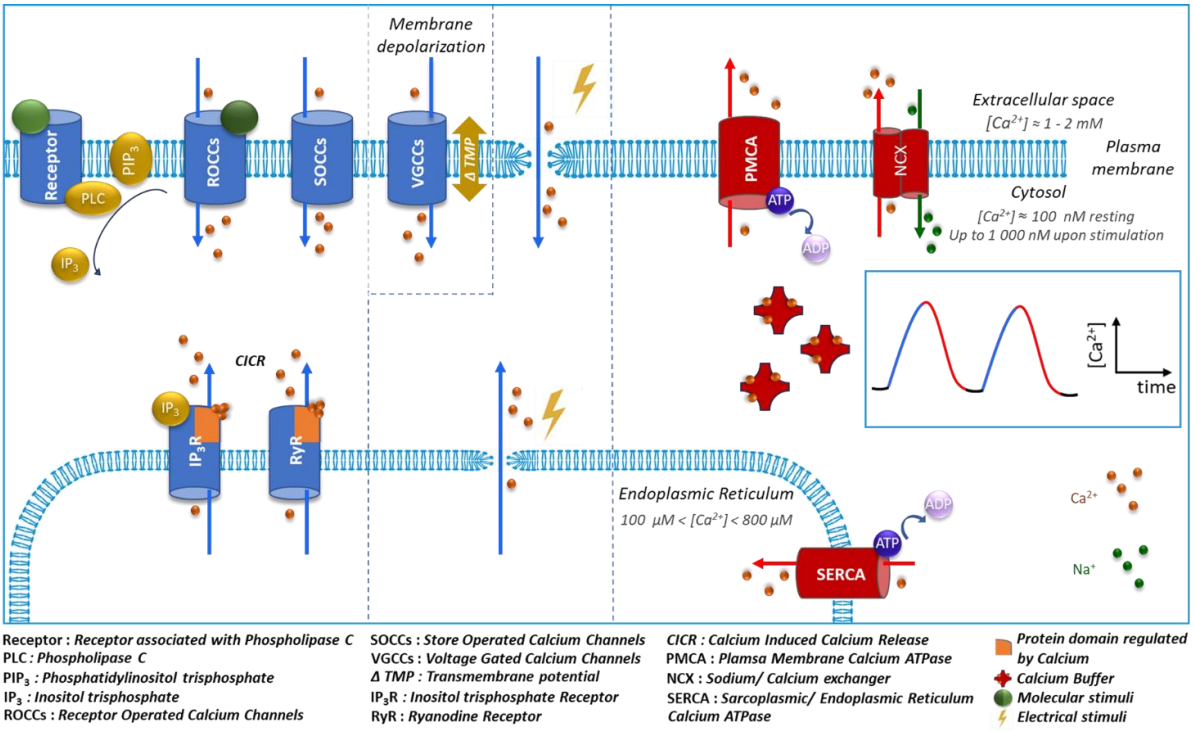

Figure 1 Mechanisms involved in the generation of cytosolic Ca^{2+} oscillations. The figure is divided into three sections by dashed lines. Left section represents actors involved in the entry of Ca^{2+} in the cytosol as a result of cellular processes, while middle section represents routes of Ca^{2+} entry into the cytosol as a result of electrical stimulation either activating VGCCs (through membrane depolarization, which can also occur as a result of cellular/intercellular processes) or causing electropermeabilization of the plasma membrane or of the membrane of the endoplasmic reticulum (intracellular Ca^{2+} store). Right section represents actors involved in the extrusion of Ca^{2+} excess from the cytosol toward the extracellular space or back to intracellular Ca^{2+} stores such as the endoplasmic reticulum. Note that blue arrows represent Ca^{2+} fluxes contributing to increase cytosolic $[Ca^{2+}]$ while red arrows represent Ca^{2+} fluxes contributing to decrease cytosolic $[Ca^{2+}]$. (Adapted from [29])

The encoded signals are subsequently decoded by a large number of proteins whose activities are modulated by Ca^{2+}, the so-called Ca^{2+} effectors. The most important Ca^{2+} effector is calmodulin (CaM), which regulates the activity of a large number of enzymes such as the Ca^{2+}/CaM-dependent protein kinase II (CaMKII) or the Ca^{2+}/CaM-dependent phosphatase calcineurin, which in turn regulate the activity of a variety of other proteins [29,31]. Information is conveyed in the frequency, amplitude, and shape of Ca^{2+} transients, directly relating to the on/off kinetics of Ca^{2+} binding to the various Ca^{2+} effectors. Some known proteins/signaling pathways that are affected by specific ranges of Ca^{2+} transient frequencies are shown in Figure 2 [31].

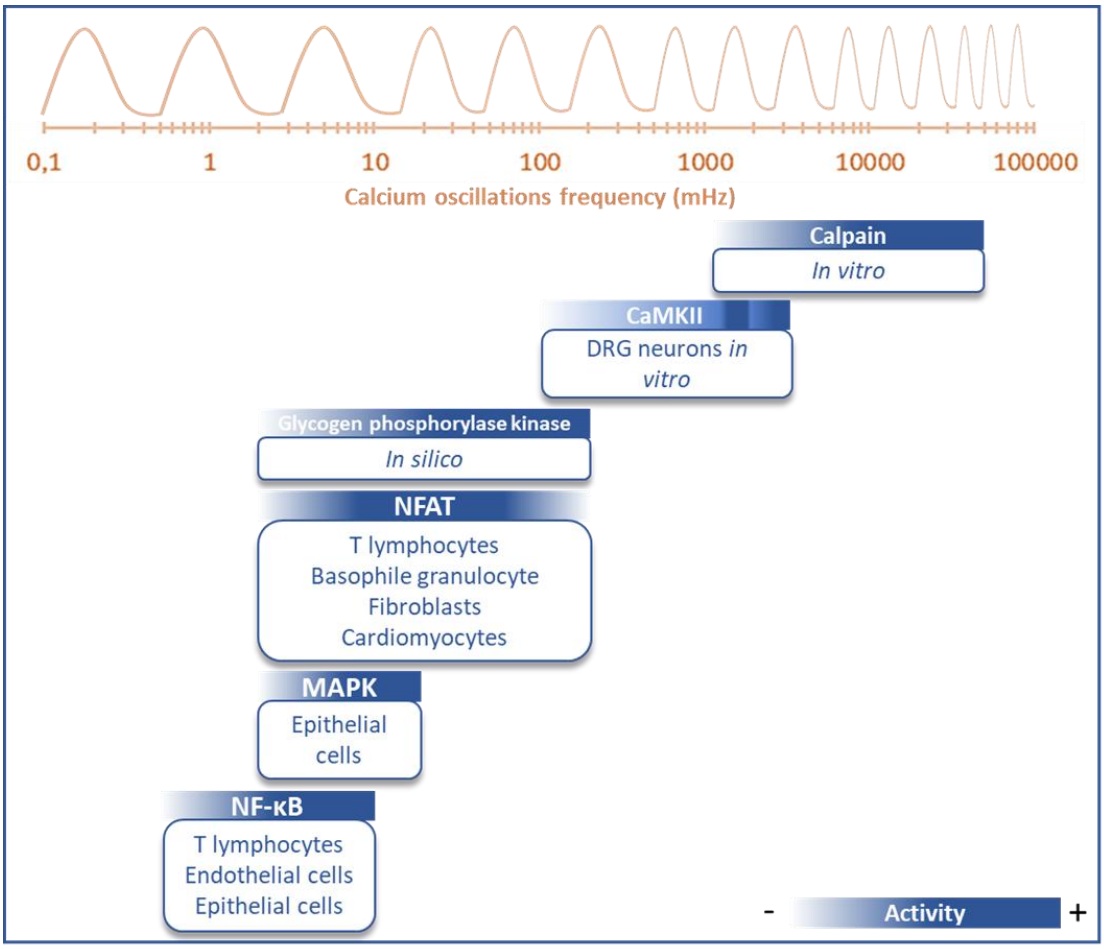

Figure 2 Ca^{2+} oscillations frequencies known to modulate the activity of different proteins (so-called frequency decoders) and host cells (or other systems) in which the modulation has been observed. Note that the scale of frequencies is in mHz (Adapted from [31])

Overall, Ca^{2+} is involved in almost all cellular processes. In excitable cells, it is a major player in action potentials (especially in cardiac action potentials in which it ensures the plateau phase) and can influence cellular excitability [32]. It is also a major player in muscle contraction [33], is key to exocytotic phenomena [34] involved in the release of vesicles containing neurotransmitters [35] or hormones [36]. Ca^{2+} has also been proposed as a mediator of cell electrotaxis [37–39]. In addition, Ca^{2+} also plays an important role in metabolism [40] and influences many cellular events related to cell fate, such as proliferation [41], differentiation [42,43], survival [44] or on the contrary, cell death [27,44,45].

Ca^{2+} and cell death

Alterations resulting from a failure to maintain Ca^{2+} homeostasis or regulated Ca^{2+} signaling can both lead to cell death [46]. Loss of Ca^{2+} homeostasis, often resulting in a sustained increase in cytosolic Ca^{2+} levels, stimulates various Ca^{2+}-sensitive catabolic enzymes, such as proteases, endonucleases, and phospholipases [27,28], producing rough damage [46]. In contrast, specific Ca^{2+} signaling can also lead to cell death in a regulated manner [47] (e.g. apoptosis of CD4 T lymphocytes)[44]. Actually, many types of cell death are associated with increased cytosolic Ca^{2+} levels: apoptosis, necroptosis, ferroptosis or

parthanatos [45]. Although increased cytosolic Ca^{2+} levels appear to be a common feature of different types of cell death, they may occur at different stages of the process and may result from different Ca^{2+} mobilizations [45], further emphasizing the importance and significance of Ca^{2+} compartmentalization in Ca^{2+} signaling. Finally, cytosolic Ca^{2+} overload can also lead to bioenergetic catastrophe [28]. This occurs when the cell exhausts its ATP in an attempt to extrude the excess Ca^{2+} and loses its ability to produce ATP [27,48,49]. This is exactly what is exploited in "Ca^{2+} electroporation" (as an anti-tumor treatment), where extracellular Ca^{2+} is elevated to supraphysiological levels to induce consequent Ca^{2+} entry through cell permeabilization [50].

Electric fields as a valuable tool to "hack" Ca^{2+} signaling

Several methods can be considered to control Ca^{2+} signaling and can be classified into different categories. Chemical methods, relatively easy to set up, rely on the use of inhibitors or enhancers of the activity of specific actors of the Ca^{2+} signaling machinery [51], or on playing with the extracellular Ca^{2+} concentration (e.g., continuous exposure to defined levels of extracellular Ca^{2+} [52–54], or Ca^{2+} clamping, which adds to the former method the depletion of Ca^{2+} from the endoplasmic reticulum (ER) to activate store-operated Ca^{2+} channels (SOCCs), allowing direct and rapid manipulation of cytosolic Ca^{2+} levels [55]. Physical methods are particularly sharp because they can be easily switched on and off as compared to, for example, the use of a specific chemical. In this perspective, we focus on the use of electric fields (EFs) to influence Ca^{2+} signaling. As mentioned above, Ca^{2+} signaling is governed by the dynamics of Ca^{2+} compartmentalization between different cell domains separated by membranes. EFs interact with membranes, altering the transmembrane potential (TMP), inducing chemical modifications (e.g. lipid oxidation), or inducing changes in protein conformation [56]. If EFs are able to depolarize the membrane to the required threshold, they can activate voltage-gated Ca^{2+} channels (VGCCs) and induce Ca^{2+} mobilization from the extracellular space into the cytosol, thereby increasing cytosolic Ca^{2+} levels [57,58]. EFs may also directly permeabilize membranes to Ca^{2+} ions [59]. Therefore, EFs are very suitable candidates to modulate Ca^{2+} signaling. Furthermore, decades of intensive research on electroporation have provided insights into the nature of permeabilization induced by specific EFs and into the membranes affected (e.g., effects of microsecond pulsed electric fields (PEFs) [59], nanosecond [60,61], or even sub-nanosecond PEFs [62] on the Ca^{2+} mobilizations and permeabilization of outer and inner cell membranes). As a result, different applications of distinct EFs can be considered. For example, the application of nsPEFs can induce apoptosis or necrosis, with the outcome depending on the external Ca^{2+} concentration [63]. The use of µsPEFs is very convenient to manipulate Ca^{2+} oscillations [64]. Exposure of cells to direct or alternative currents also affects cytosolic Ca^{2+} levels and Ca^{2+} oscillations, ultimately influencing cell fate decisions such as proliferation or differentiation [65–67]. Directly in line with the effect of AC-EFs on Ca^{2+} levels, the effect of tumor treating fields (TTFs), for which mechanistic explanations remain elusive, has been proposed to be mediated in part by Ca^{2+} [16,68].

On another note, while this perspective emphasizes Ca^{2+} signaling in mammalian cells and the potential that « hacking it » on a long-lasting basis through electrostimulation, such as MILD electroporation, could hold in terms of future biomedical applications, it is worth mentioning that Ca^{2+} signaling is also tightly regulated in yeast or plant cells for instance [69,70]. Potential applications of MILD electroporation for manipulating Ca^{2+} signaling in such organisms might also be envisioned, in analogy to conventional electroporation whose use is already established for a variety of purposes [71].

Contributing to the foundation of the field

During the 5th World Congress on Electroporation and Pulsed Electric Fields in Biology, Medicine, and Food & Environmental Technologies (WC2024) in Rome, we presented a few studies we have carried out during the last years that support the use of continuously repeated electroporation to trigger Ca^{2+} uptake.

With µsPEFs, it is possible to induce different Ca^{2+} mobilizations. At relatively low amplitudes, only the plasma membrane is permeabilized, whereas as the amplitude increases, internal membranes are also affected, e.g. with Ca^{2+} mobilization from the ER [59,64]. In the context of mesenchymal stem cells (MSCs), which exhibit spontaneous Ca^{2+} oscillations under classical culture conditions [72–74], it is possible to elicit Ca^{2+} oscillations similar to the natural ones by using µsPEFs which permeabilize the plasma membrane. This allows for the introduction of a small amount of Ca^{2+} into the cytoplasm, which is able to trigger the Ca^{2+}-induced-Ca^{2+}-release response (CICR), mobilizing Ca^{2+} from the ER [64]. CICR is due to the fact that RyR and IP_3R Ca^{2+} channels at the membrane of the ER are regulated by Ca^{2+} itself, with the binding of Ca^{2+} ions first eliciting greater activation of the channels, amplifying the $[Ca^{2+}]$ increase in the cytosol[75–77]. It is part of the natural process of Ca^{2+} oscillation in MSCs, in which the initial Ca^{2+} mobilization comes from the ER itself [72–74]. MSCs can differentiate and we found a progressive decrease of the Ca^{2+} oscillations frequencies along MSCs differentiation [78,79]. This evolution is consistent with the fact that oscillations frequencies observed in proliferating MSCs are known to efficiently activate RAS/MAPK pathway [55]. Therefore, we hypothesized that Ca^{2+} oscillation frequencies above 10 mHz tend to promote proliferation, whereas frequencies below 10 mHz tend to promote differentiation. Experimentally, the application of bipolar square µsPEFs (25 µs + 25 µs) at 300 V/cm, imposing a Ca^{2+} oscillation frequency of 17 mHz for 30 min per day for 5 consecutive days did indeed promote cell proliferation compared to unstimulated cells. This was shown by counting a greater number of cells in the stimulated conditions as compared to unstimulated control ones, for equivalent number of cells at the beginning of the experiment. These results are nicely supported by other work showing that PEFs imposing Ca^{2+} oscillations at a frequency of 33 mHz for 30 min induces the upregulation of EGR1 (Early Growth Response Protein 1) as early as 1h after stimulation and the upregulation of MKi67 (Ki-67), CCND1 (Cyclin D1) and CCNE1 (Cyclin E1) 24 h after stimulation compared to non-treated MSCs [80].

At WC2024, we highlighted recent studies showing that Ca^{2+} uptake triggered by electroporation plays a key role in cell death during conventional treatments [81,82] and that, although less frequent, pore formation can still be observed at transmembrane voltages well below the commonly cited 200 mV threshold typically considered the lower limit for electroporation [83]. More interestingly, we discussed the potential role of electroporation-induced Ca^{2+} influx in the mechanism of Tumor Treating Fields (TTFs). As mentioned above, it has been proposed that the mechanism of action of TTFs, which are continuously delivered alternating electric fields with frequencies around 100 kHz and magnitudes of approximately 3 V/cm that inhibit tumor growth, is mediated, at least in part, by Ca^{2+} [16,68]. We hypothesize that Tumor Treating Fields (TTFs) induce Ca^{2+} uptake through MILD electroporation. Based on this assumption, for a more effective treatment, we propose using alternating electric fields of higher magnitude delivered as short bursts, rather than continuously, to enhance intermittent, electroporation-mediated Ca^{2+} uptake while maintaining, or even reducing, the level of Joule heating produced by TTFs. At WC2024, we presented very preliminary *in vitro* data indicating that these burst waveforms are indeed more effective than standard TTFs in inhibiting cell proliferation.

Glossary of abbreviations, acronyms, and terms:

AC-EF: Alternating current electric field

ATP: Adenosine triphosphate

CaM: Calmodulin

CaMKII: Ca^{2+}/Calmodulin-dependent protein kinase II

CCND1: gene encoding Cyclin D1

CCNE1: gene encoding Cyclin E1

CICR: Calcium-induced calcium-release

Cyclin D1: protein involved in cell cycle regulation

Cyclin E1: protein involved in cell cycle regulation

DNA: Deoxyribonucleic acid

EF: Electric field

EGR1: gene encoding Early growth response protein 1

ER: Endoplasmic reticulum

IP3: Inositol trisphosphate

IP3R: Inositol trisphosphate receptor

Ki-67: Protein present in the nucleus of cells that are actively dividing

MAPK: Mitogen-activated protein kinases

MILD: Moderate intensity and lengthy delivery

MKI-67: gene encoding Ki-67

MSCs: Mesenchymal stem cells

NCX: Sodium/calcium exchanger

PEF: Pulsed electric field

PLC: Phospholipase C

PMCA: Plasma membrane calcium ATPase

PRF: Pulsed radiofrequency

RAS: RAS GTPase, named after rat sarcoma

RNA: Ribonucleic acid

ROCCs: Receptor operated calcium channels

RyR: Ryanodine receptor

SERCA: Sarcoplasmic and endoplasmic reticulum calcium ATPase

SOCCs: Store operated calcium channels

TTFs: Tumor treating fields

VGCCs: Voltage gated calcium channels

Authors' Contributions

L.V. contributed to conceptualization, data curation, investigation, methodology, visualization, writing—original draft and writing—review and editing. A.I. contributed to conceptualization, methodology, project administration, resources, writing—original draft, and writing—review and editing.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This work was financially supported by the Agencia Estatal de Investigación, Plan Estatal de Investigación Científica,Técnica y de Innovación under Grant PID2023-149699OB-I00. A. I. gratefully acknowledges the financial support by ICREA under the ICREA Academia programme.

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