



Plasma membrane depolarization and permeabilization due to electric pulses in cell lines of different excitability

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ABSTRACT

In electroporation-based medical treatments, excitable tissues are treated, either intentionally (irreversible electroporation of brain cancer, gene electrotransfer or ablation of the heart muscle, gene electrotransfer of skeletal muscles), or unintentionally (excitable tissues near the target area). We investigated how excitable and non-excitable cells respond to electric pulses, and if electroporation could be an effective treatment of the tumours of the central nervous system. For three non-excitable and one excitable cell line, we determined a strength-duration curve for a single pulse of 10 ns–10 ms. The threshold for depolarization decreased with longer pulses and was higher for excitable cells. We modelled the response with the Lapicque curve and the Hodgkin-Huxley model. At 1 μ s a plateau of excitability was reached which could explain why high-frequency irreversible electroporation (H-FIRE) electroporates but does not excite cells. We exposed cells to standard electrochemotherapy parameters ($8 \times 100 \mu$ s pulses, 1 Hz, different voltages). Cells behaved similarly which indicates that electroporation most probably occurs at the level of lipid bilayer, independently of the voltage-gated channels. These results could be used for optimization of electric pulses to achieve maximal permeabilization and minimal excitation/pain sensation. In the future, it should be established whether the in vitro depolarization correlates to nerve/muscle stimulation and pain sensation in vivo.

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1. Introduction

Short, high-voltage pulses increase the permeability of cell membranes to different molecules (reversible electroporation) or cause cell death (irreversible electroporation) [1–3]. Electroporation is used in biotechnology, food-processing [4–6] and medicine [7], e.g. gene electrotransfer [8–10], DNA vaccination [11–14], transdermal drug delivery [15,16], IRE as a soft tissue ablation technique [17–20] and electrochemotherapy [21–24].

In medical applications of electroporation, different types of tissues and tumours are treated, among them, also excitable tissues, e.g. critical structures like nerves and spine. Non-excitable as well as excitable cells

can be depolarized, i.e. their transmembrane potential increases. However, only excitable cells can produce action potentials due to their expression of a high density of voltage-gated channels which enables electrical communication between cells [25]. Types of excitable cells include neurones, muscle, endocrine and egg cells.

In the literature, there are several examples of electrochemotherapy, irreversible electroporation and gene electrotransfer of excitable tissues by electric pulses. Brain cancer is treated with irreversible electroporation, and electric pulses can transiently disturb the blood-brain-barrier and allow chemotherapeutics to enter the brain [26–31]. Treating prostate cancer can affect the neurovascular bundle [32,33], treating bone metastases can affect nerves [21,34], treating tumours in the spine can affect the spinal cord [34]. When treating tumours in other parts of the body, electrodes will invariably be in the vicinity of the nerves or muscles where the electric field is high enough for excitation or even permeabilization. Electric pulses are also used for ablation of myocardial tissue to treat atrial fibrillation [35–37]. Muscles are a popular target for gene electrotransfer as they are easily accessible and transfected [38,39]. Among them, the heart can be electroporated to treat ischemia [40,41]. Other examples of the application of electric pulses to excitable cells include electroporation of neurons as a labelling technique which

Abbreviations: ECT, electrochemotherapy; IRE, irreversible electroporation; CHO cells, Chinese hamster ovary cells; MEM, minimal essential medium; DMEM, Dulbecco's minimal eagle's medium; PMPI, plasma membrane potential indicator; YP, YO-PRO-1®.

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enables subsequent analyses [42–46]. Studies exist on the influence of nsPEF on intracellular calcium and release of catecholamine in endocrine cells [47–49].

Several studies showed that the effect of electric pulses on the functionality of excitable tissues was only short-term. After irreversible electroporation the nerves of different animal models recovered electrophysiologically, histologically and functionally [34,50–52] or at least showed a potential for regeneration [53]. After electroporation of individual rat neocortex neurons, *in vitro* and *in vivo*, the membrane potential, the action potential waveform and passive membrane properties remained unchanged [54]. After pulmonary vein ablation with electroporation, the histology and functionality of phrenic nerve remained unchanged [55]. There was no histological damage on nerves in the neurovascular bundle when treating prostate with irreversible electroporation [51].

One of the main drawbacks to the treatment of tissues with pulsed electric fields is the discomfort and pain associated with repeated electrical stimulation [56–59], the need to administer muscle relaxants and anaesthesia [60] and synchronization with the electrocardiogram [61–63]. The neurons responsible for pain sensation (nociceptors) can be stimulated by electric pulses [64,65]. An important advance of the treatment would be to determine a point at which maximum permeability of the membrane could be achieved while minimizing excitation of the exposed excitable cells. In electrochemotherapy, for example, this would translate to maximum drug delivery to tumour cells with minimum tissue damage to surrounding regions, reduced pain experienced by the patient, and minimal use of muscle relaxants. One suggested option is applying short bipolar pulses which do not cause muscle contraction but come at the expense of delivering higher energy than with longer monopolar pulses [66].

Questions that need to be answered include, (i) whether excitable and non-excitable tissues respond similarly to electroporation pulses, (ii) can electroporation be an effective treatment of cancers of the central nervous system, (iii) are the properties of surrounding excitable tissues significantly altered or damaged due to the treatment. In our paper, we evaluated the depolarization and membrane permeability of four cell lines, one excitable and three non-excitable. For each cell line, we determined the strength-duration curve to one pulse of durations between 10 ns and 10 ms. Additionally, response of excitable cell line was modelled with the Lapicque curve and the Hodgkin-Huxley model. Lapicque curve is an empirical description of the strength-duration curve and the Hodgkin-Huxley model is a set of differential equations, describing the dynamics of voltage-gated channels when they are exposed to electric stimulus. Our study shows that higher electric fields are needed for depolarization of excitable cell line than for depolarization of non-excitable cell lines. We compared the depolarization results with previously experimentally determined electroporation results modelled with the Saulis model [67]. We explain the lack of excitation after electroporation with short 1 μ s pulses as well as high frequency short bipolar pulses (H-FIRE). This lack of excitability with short bipolar pulses can be explained by reaching a plateau of excitation and electroporation around 1 μ s. We also determined the permeability curve to pulses of standard electrochemotherapy parameters ($8 \times 100 \mu$ s, 1 Hz at different electric field amplitudes). All four cell lines were permeabilized at approximately the same electric field, and the permeabilization curves were similarly shaped which indicates that electroporation is independent of voltage-gated channels.

2. Materials and methods

2.1. Cell culture/preparation

Four cell lines of different excitability were used (Fig. 1). CHO Chinese hamster ovary cells (European Collection of Authenticated Cell Cultures ECACC, CHO-K1, cat. no. 85051005, obtained directly from the repository), U-87 MG human glioblastoma cells (ECACC, Public

Health England, cat. no. 89081402), and HT22 immortalized mouse hippocampal neurons (The Salk Institute, La Jolla, CA) were grown in an humidified environment at 37 °C at 5% CO₂. CHO cells were grown in the Ham-F12 (Sigma-Aldrich, Germany), U-87 MG in MEM (Sigma-Aldrich, Germany) and HT22 cells in DMEM (Sigma-Aldrich, Germany) growth medium. All growth media were supplemented with 10% fetal bovine serum, L-glutamine and antibiotics. Cells were grown either on Poly-L-lysine (Sigma-Aldrich, Germany) coated 22 mm glass coverslips which were put inside a plastic ring or on 40 mm Petri dishes (TPP, Austria). The HT22 cell line was differentiated by 48 h incubation in the Neurobasal medium (Gibco, USA) supplemented with 0.5 mM L-glutamine and B-27 serum-free supplement as advised by the manufacturer. In this time, the cells stopped dividing and changed their morphology (Fig. 1c vs d). We tested the excitability by chemically exciting the cells by varying the concentration of extracellular potassium ions and comparing the response to a 1 ms pulse at 0.6 kV/cm.

2.2. Cell labelling – depolarization and membrane permeability

In the depolarization experiments, we labelled the cells with the plasma membrane potential indicator fluorescent dye (PMPI) of the FLIPR Membrane Potential Assays Kit (Molecular Devices, USA). For 30 min before the experiments, cells were incubated at 37 °C and 5% CO₂ in Live Cell Imaging Solution (Fischer Scientific, France) supplemented with 1 mg/ml 20% D-glucose (Gibco, France) and 0.5 μ l/ml PMPI. PMPI consists of a two-part system which includes a fluorescent anionic voltage-sensor and a quencher. When the interior of the cell has a relatively negative charge (in the state of resting potential or repolarization), the anion dye remains bound to the external surface of the plasma membrane. In this state, the quencher in the extracellular fluid prevents fluorescence excitation. During depolarization the voltage sensor translocates to the interior of the plasma membrane. This translocation is reversible as we observe cells return to their base-level fluorescence approximately 30 min after exposure to pulse. An increase in fluorescence is observed because the quenching agent is restricted to the extracellular environment. The time-constant of sensor translocation is in the range of seconds [68].

To determine whether cells were excitable, cells were exposed to different extracellular concentrations of potassium ions (2.5 mM, 25 mM, 50 mM, 75 mM, 100 mM, 140 mM), while the NaCl (140 mM in the original Live Cell Imaging Solution) was substituted for KCl in an equimolar manner. The buffer was changed every 5 min, and the cells were continuously imaged. Increased concentration of extracellular K⁺ ions increases the equilibrium potential of K⁺ and causes depolarization. Chemical depolarization is slow and does not cause action potential but accommodation. In electrical depolarization experiments, images were acquired every 30 s for 15–30 min, and pulse was delivered 5 min after the beginning of imaging.

In permeability experiments, we labelled the cells with the YO-PRO-1® (YP) (Molecular Probes, USA). Right before the experiments, the growth medium was changed with the Live Cell Imaging Solution supplemented with 1 mg/ml 20% D-glucose (Gibco, France) and 1 μ M YP. Images were acquired every 3 s for 6 min, and $8 \times 100 \mu$ s pulses were delivered 30 s after the beginning of the imaging.

2.3. Exposure of cells to electric pulses

Three different pulse generators were used, each for a different pulse duration range. For 10 ns pulses, we used a commercially available nsPEF (nanosecond pulsed electric fields) generator (FPG 10-1NM-T, FID Technology, Germany) where the electric field was numerically determined [69] (Fig. 3a). For pulse exposures of 550 ns–1 μ s, we used a laboratory prototype pulse generator (University of Ljubljana) based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA) [70] (Fig. 3b). For pulse exposures of 10 μ s–10 ms we used a commercially available BetaTech electroporator (Electro cell B10,

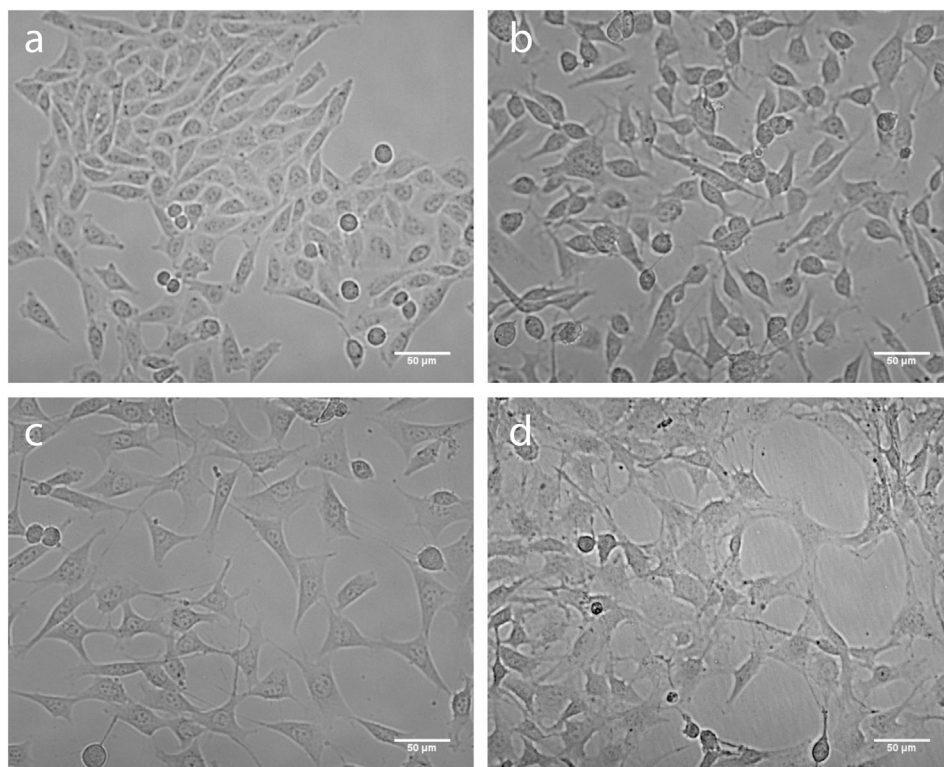


Fig. 1. Phase-contrast images of all four cell lines used in experiments. a - CHO, b - U-87 MG, c - undifferentiated HT22 and d - differentiated HT22.

BetaTech, France). In all experiments, we measured the delivered voltage and current by oscilloscope (DPO 4104, Tektronix, USA or Wavesurfer 422, 200 MHz, LeCroy, USA), voltage probe (tap-off 245 NMFFP-100, Barth Electronics Technology, USA for 10 ns pulses or differential probe ADP305, LeCroy, USA for longer pulses) and a current probe (CP030, LeCroy, USA or Pearson current monitor model 2877, Pearson Electronics, Inc., USA). We used different systems as not all pulse generators were available in both laboratories.

For 10 ns pulses, we used two stainless-steel needle electrodes with a 1.2 mm gap (Fig. 2a) [69]. For pulses longer than 10 ns we used either stainless-steel wire electrodes with a 4 mm inter-electrode distance or Pt/Ir wire electrodes with 1 mm, 2.2 mm or 5 mm inter-electrode distance (Fig. 2b) [70], depending on the electric field needed and the power limitations of the generators. The electric field in the middle between the electrodes was nearly homogeneous and could be

approximated as the applied voltage divided by the distance between the electrodes [70].

In depolarization experiments, we delivered one pulse, and we varied the pulse duration (10 ns, 550 ns, 1 μ s, 10 μ s, 100 μ s, 1 ms, 10 ms), and voltage to determine the minimum electric field intensity required to cause a change in membrane potential (depolarization) that was statistically different from control conditions. This minimum electric field intensity was considered to be the depolarization threshold. In membrane permeability experiments, we delivered $8 \times 100 \mu$ s pulses at 1 Hz repetition frequency.

2.4. Fluorescence microscopy and measurement

Imaging was performed on two different fluorescent microscopy systems due to different availability of pulse generators in the two

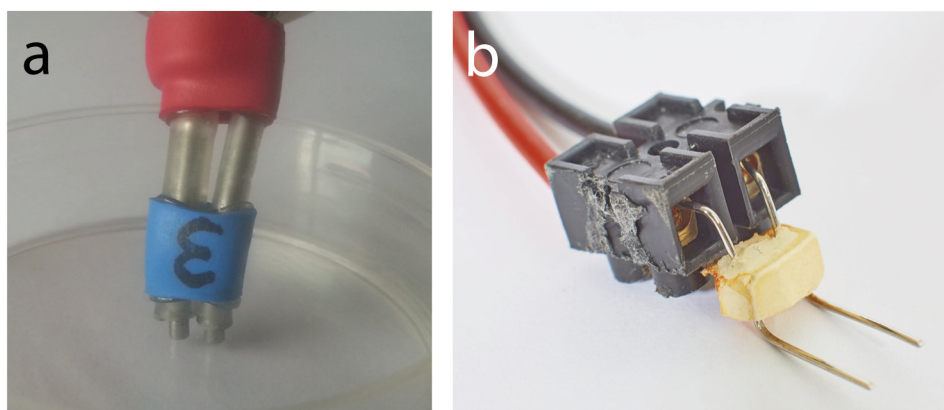


Fig. 2. Photos of the electrodes, used in our experiments. a - electrodes used for delivering 10 ns with 1.2 mm gap, b - electrodes used for delivering pulses longer than 10 ns with 5 mm inter-electrode distance.

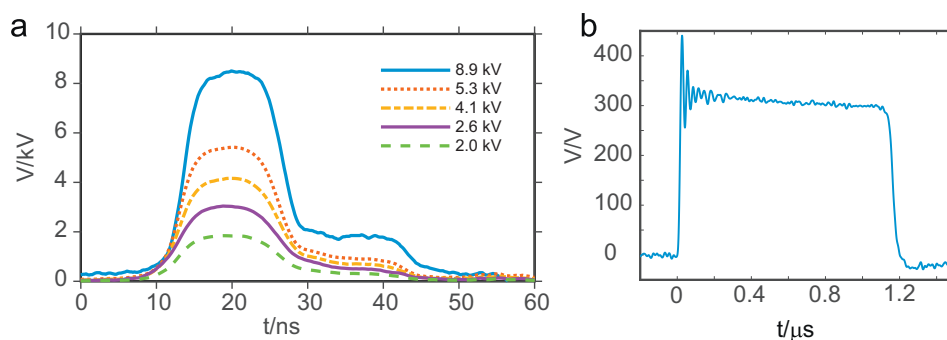


Fig. 3. Traces of pulses applied in the experiments. a - traces of 10 ns pulses generated by a commercial 10 ns nsPEF generator [69], b - a trace of 1 μ s pulse generated by a prototype generator from University of Ljubljana [70]. Voltage V/kV or V/V is presented as a function of time t/ns or t/ μ s, a and b, respectively.

laboratories where the work was done. However, by repeating several samples on both systems, we determined that the difference in systems did not influence the results. Either a DMI6000 inverted microscope (Leica Microsystems, Germany) with EMCCD camera (EMCCD Evolve 512, Roper, USA) or an AxioVert 200 inverted microscope (Zeiss, Germany) with VisiCam 1280 CCD camera (Visitron, Germany) and either a 100 $\times$ oil immersion or 40 $\times$ dry objective for PMPI and 20 $\times$ dry objective for YP were used for experiments. Samples were excited with appropriate wavelengths using the Spectra 7 light engine (Lumencor USA) or a monochromator (High-Speed Polychromator, Visitron Systems GmbH, Germany) and the emission light was selected through appropriate filters. Images were acquired using MetaFluor and MetaMorph PC software (both Molecular Devices, USA).

2.5. Image analysis

First, the background was subtracted, each cell was selected by using freehand tool, and its mean fluorescence was determined. In

depolarization experiments, we determined the maximal fluorescence in the first 2.5 min and normalized it to the base line (the value of fluorescence prior exposure to pulses). In permeability experiments, the cells do not take up any YP without the electric pulses applied. Thus, we reported the values either raw or normalized to the fluorescence at maximal pulse parameters (8 $\times$ 100 μ s, 1.2 kV/cm, 1 Hz repetition frequency).

2.6. Statistical analysis

The threshold of depolarization was determined using ANOVA tests. The threshold was determined as the lowest field intensity required to produce a statistically significant membrane depolarization. The statistical parameters are given in the Appendix, Table A1. The threshold of electroporation (the fluorescence that was significantly different from the control) and the comparisons of the change in maximal fluorescence between all four cell lines were determined using *t*-test ($p < 0.05$) in SigmaPlot v.11 (Systat Software, San Jose, CA).

3. Calculation/models

3.1. Cell excitability

Cell excitability models described the depolarization/action potential thresholds of the excitable differentiated HT22 cell line as the models we used are valid for excitable cells. We modelled the strength-duration curve with two models – the Lapicque curve [71] which is one of the most often used experimentally tested theoretical model and the Hodgkin-Huxley model [72] which is a phenomenological description of the activity of voltage-gated channels.

The Lapicque curve is in the form:

$$I = b \left(1 + \frac{c}{T} \right), \quad (1)$$

where *b* denotes the rheobase, *c* the chronaxie and *T* the duration of the stimulus. Since we were controlling the electric field to which cells were exposed, we substituted the current (*I*) with the electric field (*E*). For the rheobase value we took the depolarization threshold at applied 10 ms, i.e. 0.28 kV/cm. The chronaxie should thus be the pulse duration at twice the rheobase, i.e. around 6 μ s. However, with these parameters, the curve fits the data poorly, and thus we determined the parameter *c* to be 1.88 μ s. A non-physiological value of parameter indicates that the Lapicque curve is not an optimal choice for description of our data.

One of the classical models describing neuronal excitation is the Hodgkin-Huxley (HH) model. We numerically calculated the strength-duration curve via the HH model as described in [71]. For several pulse durations, we calculated the corresponding critical transcellular voltage which triggers the action potential (Fig. 4). The K, Na, and leakage current were modelled separately for the anodic and cathodic pole of the cell and coupled via the equivalent circuit (Fig. 1A in [71]). Cells were modelled as idealized planar cells with two uniformly polarized flat surfaces. The corresponding external electric field was calculated by dividing the transcellular voltage with the diameter of the cell. We modelled monopolar as well as bipolar pulses. The bipolar pulse consisted of a positive pulse immediately followed by a negative pulse. The duration of the bipolar pulse is the duration of a separate positive or negative pulse – the whole duration was thus twice this value. The cell excitability calculations were performed in Matlab, R2017a (Mathworks, USA). Shape of the strength-duration curve depends on the time constant of the membrane (Fig. 3 in [71]).

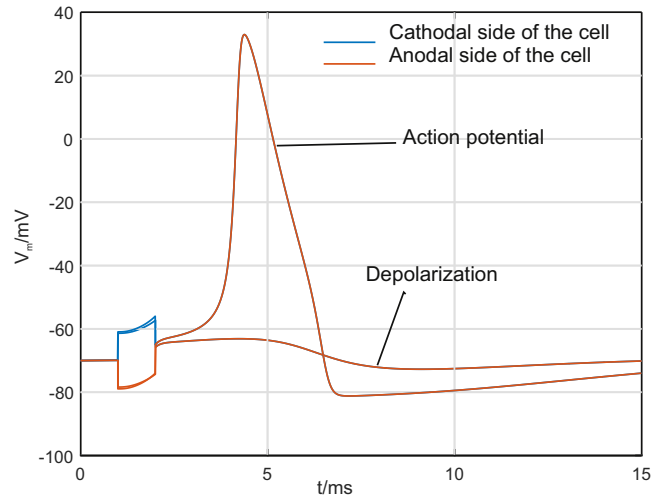


Fig. 4. Example of depolarization without and with initiated action potential. Blue and orange lines denote the voltage at the cathodal and anodal pole of the cell, respectively. After the stimulus, only the orange line is visible as the blue and orange lines are one on top of another and overlap. In this example, one 1 ms pulse was applied just below and at the threshold. Induced transmembrane voltage V_m /mV is presented as a function of time t /ms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Plasma membrane permeabilization

Permeabilization consisted of two sets of experiments – those with the application of one pulse (results previously published in [67]) and the application of 8 pulses (data acquired in this study).

When one pulse was applied, two models were used – a time-dependent Schwann equation or theory of kinetics of pore formation. First, we used the time-dependent Schwann equation for prolate ellipsoidal cells [73,74]:

$$\Delta V_{crit} = E \frac{R_1^2 - R_2^2}{R_1 - \frac{R_2^2}{\sqrt{R_1^2 - R_2^2}} \log \left(\frac{R_1 + \sqrt{R_1^2 - R_2^2}}{R_2} \right)} \frac{R_2 \cos(\varphi)}{\sqrt{R_1^2 \sin^2 \varphi + R_2^2 \cos^2 \varphi}} \left(1 - \exp \left(-\frac{t}{\tau} \right) \right), \quad (2)$$

where the time constant of the membrane was $0.87 \mu\text{s}$ (τ) as determined in [67] and $R_1 = 27 \mu\text{m} \pm 7 \mu\text{m}$ and $R_2 = 10 \mu\text{m} \pm 5 \mu\text{m}$ which we determined from 131 differentiated HT22 cells on phase-contrast images. The critical threshold of electroporation (V_{crit}) 1.776 V was obtained by the least-square method, but the fit was poor ($R^2 = 0.53$). Parameter t denotes time and φ the angle between the electric field and position on the plasma membrane. Because we were interested in the maximal transmembrane voltage, we assumed $\varphi = 0$ (prolate ellipsoid oriented in the direction of the electric field. Electric field (E) was expressed and we calculated the critical applied electric field for electroporation.

Second, we used the expression for a fraction of electroporated cells as derived by [75]. We used the optimized parameters for attached cells in a monolayer from (Eq. (5) and Fig. 5 in this [67]):

$$F_p(E, t_p) = 1 - \exp(-k_f(E)t_p) \quad (3a)$$

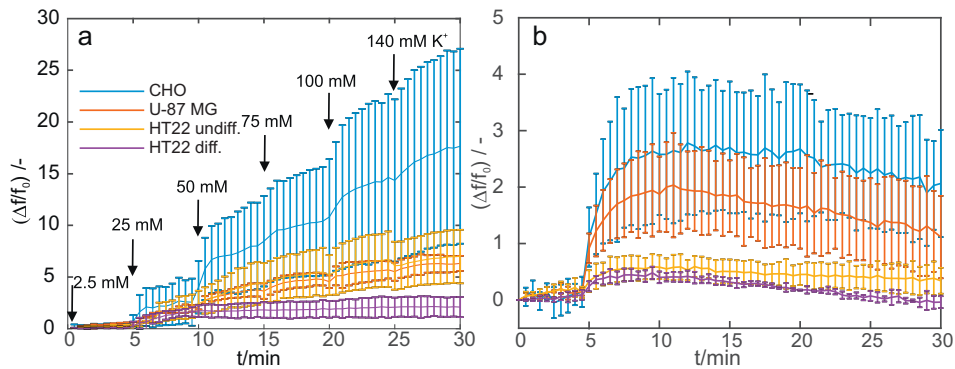


Fig. 5. Time-dynamics of electrical and chemical depolarization of all four cell lines. a) Chemical depolarization for all four cell lines. The K^+ concentration was increased every 5 min. The arrows mark which K^+ concentration cells were exposed to from that time point further. b) Electrical depolarization when 1 pulse of 1 ms and 0.6 kV/cm was applied. In a and b the increase over base level $(\Delta f/f_0)$ /arbitrary units is presented as a function of time t /min with shown 1 standard deviation.

$$k_f(E) = \frac{2\pi\nu R^2}{a} \exp\left(-\frac{\Delta W_0}{k_b T}\right) \int_{-\pi}^{\pi} \exp\left[\pi C_m r^2 \frac{(\epsilon_w - 1)}{2k_b T} \left(K_1 E R \cos(\theta) \left(1 - \exp\left(\frac{-t_p}{K_2 \tau}\right)\right) + \Delta\varphi\right)^2\right] d\theta, \quad (3b)$$

where F_p denotes the fraction of electroporated cells (0.7), t_p the duration of the pulse, ν the frequency of lateral fluctuations of the lipids (10^{11} s^{-1}), R is by fitting determined cell radius (15.9 μm) [67], a the area per lipid molecule (0.6 nm^2), ΔW_0 the energy barrier for pore formation (46.4 $k_b T$), k_b the Boltzmann constant, T the temperature in kelvins, r the radius of the pore (0.32 nm), C_m the capacity of the membrane (1 $\mu\text{F}/\text{cm}^2$), ϵ_w (78) and ϵ_m (4) the relative permittivity of the water in the pore and membrane, respectively, K_1 (1.24) and K_2 (2.56) two numerical parameters, τ the membrane time constant (0.87 μs), $\Delta\varphi$ resting membrane voltage (−25 mV). The values were used as optimized by experimental data on attached CHO cells and electroporation was determined by Fura-2AM [67].

When 8 pulses were applied, the normalized fluorescence curve was obtained by:

$$f_n(E) = \frac{f(E) - f(E = 0 \text{ kV/cm})}{f\left(E = 1.2 \frac{\text{kV}}{\text{cm}}\right) - f(E = 0 \text{ kV/cm})} \quad (4)$$

where $f_n(E)$ denotes the normalized fluorescence, $f(E)$ the raw fluorescence and E the applied electric field. The normalized fluorescence curve was then fitted using a symmetric sigmoid to obtain permeabilization curve [76]:

$$p(E) = \frac{1}{1 + \exp\left(-\frac{E - E_{50\%}}{b}\right)} \quad (5)$$

where p denotes the normalized permeabilization, E the applied electric field, $E_{50\%}$ the electric field where 50% of the final fluorescence was reached and b the width of the curve. Permeabilization curve is based on normalized fluorescence of the population and not the exact percentage of permeabilized cells.

The raw values of YO-PRO-1-® fluorescence were described using a first-order uptake model, which gave information on the resealing speed [77]. The model was:

$$f(t) = S \left(1 - \exp\left(-\frac{t}{\tau}\right)\right) \quad (6)$$

where S is the reached plateau, t is the time, f is the raw fluorescence and τ is the time constant of the resealing when 63% of the permeable structures in the membrane are resealed. A higher value of τ means slower resealing than lower value of τ . For curve fitting, we used Matlab R2015 (Mathworks, USA) and the Curve Fitting Toolbox. The goodness of the fit was evaluated using R-squared value, whose value closer to 1 indicates better fit.

4. Results

Results are divided into two sections – cell depolarization and plasma membrane permeabilization after one pulse and plasma membrane permeabilization after pulses of standard electrochemotherapy parameters. Results in the cell depolarization section were obtained with the PMPI dye. Results in the plasma membrane permeabilization section were obtained with the YO-PRO-1® dye.

4.1. Cell depolarization and plasma membrane permeabilization after one pulse

Fig. 5 shows the response of excitable and non-excitable cells to electrochemical and electrical depolarization. In Fig. 5a, the differential behavior of cells to depolarization by increasing extracellular K^+ concentrations is shown. In Fig. 5b, the results to electrical depolarization after 1 pulse of 1 ms (the most relevant point for neurostimulation) at 0.6 kV/cm is shown. We compared the intensity of the response and thus exposed all four cell lines to electric pulses of the same parameters. The increase in fluorescence was observed when the pulse was applied at threshold or above-threshold electric fields. We also tested several different pulse durations and electric fields. Depolarization curves for other pulse durations were similar in shape and values as the curves after excitation with 1 ms pulse.

Fig. 6 shows the strength-duration depolarization curve (right y-axis) and strength-duration permeabilization curve (left y-axis). Both aspects (depolarization and membrane permeabilization) were experimentally determined as well as modelled. The experimental depolarization threshold was determined from the membrane depolarization curves, for each pulse duration and cell line separately. It is shown in

grey symbols – x denotes the CHO cells, Δ the U-87 MG cells, $\square$ the undifferentiated HT22 cells and $\circ$ the differentiated HT22 cells. The exact values are stated in Table 1. We can see that with increasing pulse

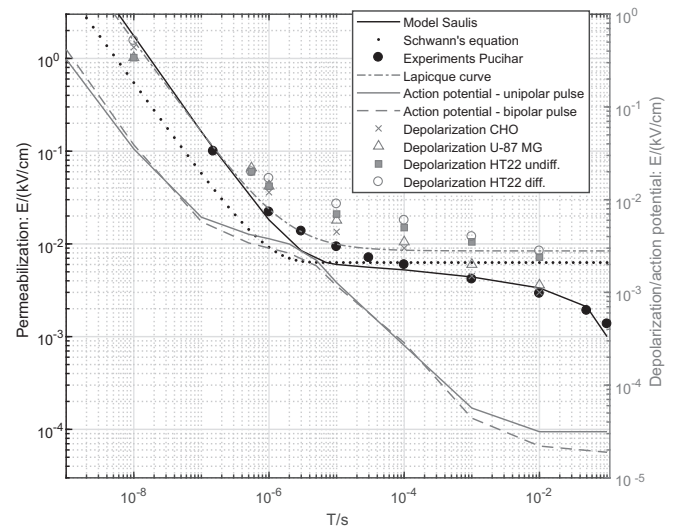


Fig. 6. The depolarization strength-duration curve for CHO, U-87 MG, undifferentiated and differentiated HT22 cells and permeabilization curve from [67] for CHO cells. Black lines and left y-axis show permeabilization to a single pulse of duration T/s . Grey lines and right y-axis show depolarization and action potential due to the application of a single pulse of duration T/s . Depolarization was determined using the PMPI dye and the electroporation by the Fura-2AM dye. Lines correspond to models and symbols to experimental values. External electric field $E/(\text{kV}/\text{cm})$ is presented as a function of pulse duration T/s .

Table 1

The depolarization thresholds for all tested pulse durations and cell lines. The results are the same as in the Fig. 6. The asterisk (*) denotes that the threshold is only estimated due to the variability of the data.

Electric field (kV/cm)	10 ns	550 ns	1 μ s	10 μ s	100 μ s	1 ms	10 ms
CHO	44	2.0	1.2	0.45	0.30	0.15	0.10
U-87 MG	34	2.2	1.4	0.60	0.35	0.20	0.12
Undifferentiated HT22	34	2.0	1.4	0.70	0.50	0.35	0.24
Differentiated HT22	52	2.0	1.7*	0.90	0.60	0.40	0.28

duration the depolarization threshold decreased. At most of the pulse durations, the threshold for depolarization was the highest for the differentiated HT22 cell line. The statistical parameters of the analysis of the experimental depolarization threshold are shown in the Appendix in Table A1. The depolarization strength-duration curve was modelled using the Lapicque curve and the Hodgkin-Huxley model. The models are only valid for excitable cell lines which have voltage-gated channels. In excitable cells we obtained excitability strength-duration curve and in non-excitable cell lines we obtained depolarization strength-duration curve. The modelled curves are presented with grey lines – in dash-dot line is the Lapicque curve, in solid line is the Hodgkin-Huxley model for the unipolar pulses and in dashed line is the Hodgkin-Huxley model for bipolar pulses. The Hodgkin-Huxley model predicts a plateau at around 1 μ s and is also slightly better at describing the data than the Lapicque curve.

Experimental electroporation thresholds are shown in filled black circles [67]. By the Saulis model modelled electroporation threshold is presented in black lines. The dashed line is the Schwann's equation. Both models follow a similar dynamics up to 1 ms, but for longer pulses, the Saulis model predicts lower electroporation thresholds and matches the experimental data better. In the Schwann model, the threshold of electroporation was assumed to be constant for all pulse lengths, although it is possible that it changed. The Saulis model was obtained by fitting the model to the experimental results [67] and thus followed the experimental data better than the Schwann equation. Depolarization and electroporation thresholds follow a similar dependency, although the electroporation thresholds are slightly higher than the depolarization thresholds. The same dependency indicates that electroporation and depolarization behave similarly as a function of the applied electric field.

4.2. Plasma membrane permeability after pulses of standard electrochemotherapy parameters

In plasma membrane permeability experiments, we delivered $8 \times 100 \mu$ s pulses of different voltage at 1 Hz repetition frequency. These parameters are the standard electrochemotherapy parameters and were used to evaluate the possibility of using electrochemotherapy

Table 2

Parameters of the fitted symmetric sigmoid to the normalized data of YO-PRO-1® uptake. $8 \times 100 \mu$ s pulses were delivered at repetition frequency 1 Hz. The numbers denote the optimal value and the corresponding 95% confidence intervals.

Cell line	$E_{50\%}$ (kV/cm)	b (kV/cm)	R-squared
CHO	0.80 ± 0.06	0.15 ± 0.11	0.98
U-87 MG	0.81 ± 0.10	0.13 ± 0.09	0.99
HT22 undifferentiated	0.94 ± 0.07	0.12 ± 0.06	0.99
HT22 differentiated	0.91 ± 0.07	0.12 ± 0.06	0.99

as treatment of excitable tissues or in the vicinity of excitable tissues. The normalized plasma membrane permeabilization curve (Eq. 4) to YO-PRO-1® is for all four cell lines shown in Fig. 7. The threshold of electroporation of the U-87 MG and CHO cells was 0.4 kV/cm while of the undifferentiated and differentiated HT22 cells it was 0.6 kV/cm. The threshold was determined as electric field intensity where the fluorescence 6.5 min after pulse application was significantly higher than the fluorescence of the control. With the increase of the electric field, the permeabilization also increased in a similar way for all four cell lines. The permeabilization curve could be described using a symmetric sigmoid (Eq. 5), which is shown in Fig. 7b. The symmetric sigmoid parameters (Table 2) show that all four cell lines reached 50% permeabilization between 0.8 and 0.9 kV/cm ($E_{50\%}$).

An example of the time dynamics of the uptake at applied $8 \times 100 \mu$ s at 1.2 kV/cm is shown in Fig. 8a. From the time-lapse of the YO-PRO-1® uptake, we obtained the maximal fluorescence. The maximal fluorescence (Fig. 8b) was the highest for the U-87 MG cells and the lowest for the CHO cells (CHO vs U-87 MG is just significant at $p = 0.0468$, CHO vs. undifferentiated HT22 $p = 0.0002$, CHO vs. differentiated HT22 $p = 0.0008$). There were no significant differences in the maximal fluorescence between the differentiated and undifferentiated HT22s while for all other pairwise comparisons the difference was significant (t -test, $p < 0.05$). We obtained the resealing constant (τ) (Fig. 8c) by fitting a first-order uptake model (Eq. 6). The value of time constant τ (Fig. 8c) corresponds to the time when 63% of the pores in the membrane resealed. The resealing was the fastest for the U-87 MG cells, similar for the undifferentiated HT22s and CHO and slower for the differentiated HT22 cells. Statistical significance of the fit is obtained by comparing the error bars which represent a statistical error of 5%.

5. Discussion

Our study we aimed at comparing the depolarization thresholds between excitable and non-excitable cells, to determine if excitable and non-excitable cells respond similarly to electroporation pulses, and to determine if electroporation can be an effective treatment of cancers of the central nervous system. For each cell line, we determined the strength-duration curve to one pulse of lengths between 10 ns and

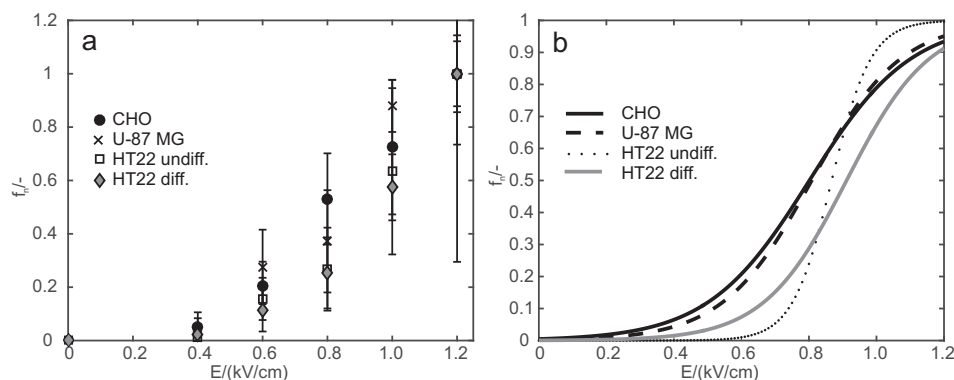


Fig. 7. Normalized fluorescence (a) and permeabilization curve (b) of all four cell lines to YO-PRO-1®, 5 min after the pulse application. The threshold for electroporation was reached at 0.4 kV/cm (U-87 MG and CHO) or 0.6 kV/cm (undifferentiated and differentiated HT22 cells). a) Normalized values, the bars are one standard deviation and b) the fitted symmetric sigmoid. Normalized fluorescence $f_n/-$ is presented as a function of applied electric field E /(kV/cm).

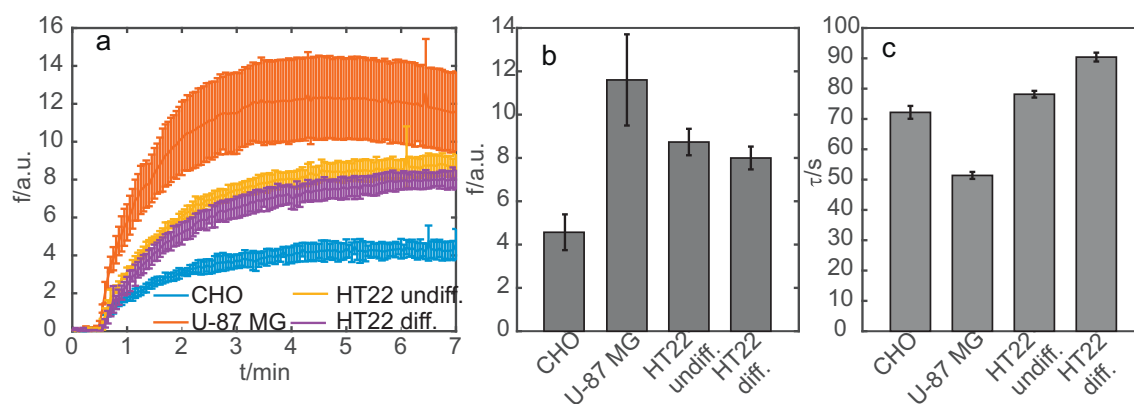


Fig. 8. Time dynamics of the YO-PRO-1® (YP) uptake and its analysis. a - Time dynamics of the YP uptake of all four cell lines at 1.2 kV/cm, $8 \times 100 \mu\text{s}$, 1 Hz repetition frequency. One bar means one standard deviation. Fluorescence f/arbitrary units is presented as a function of time t/min. b - The maximal value of YP fluorescence for all four cell lines at applied $8 \times 100 \mu\text{s}$ pulses of 1.2 kV/cm, 1 Hz repetition frequency. Value of maximal fluorescence f/arbitrary units is shown for each cell line. c - The resealing constant when $8 \times 100 \mu\text{s}$ pulses at 1.2 kV/cm are applied to all four cell lines. Value of time constant of resealing τ/s is presented for each cell line. Each error bar represents 95% confidence interval of the fitted optimal value of the resealing constant. The R-squared value was in all fits above 0.98.

10 ms. We modelled the strength-duration curve of excitable cells by the Lapicque curve and the Hodgkin-Huxley model. We compared the excitability results with permeabilization to 1 pulse of durations between 150 ns and 100 ms, previously experimentally determined and modelled using the Saulis model [67]. We also determined the permeability curve to $8 \times 100 \mu\text{s}$ pulses, delivered at 1 Hz and different electric field intensities. For the assessment of cell depolarization we used the PMPI dye. For the assessment of the plasma membrane permeabilization we used the YO-PRO-1® (YP) dye. Thus, we assume all results obtained with the dye PMPI to be depolarization due to voltage-gated channels opening or formation of the pores in the membrane. We assume all results obtained with the YP to be due to influx of YP through the voltage-gated channels or through permeable plasma membrane. Our methodology does not allow us to make distinction between molecules entering through voltage-gated channels or through pores/defects in the cell membrane.

The dye PMPI of the FLIPRR Membrane Potential Assay Kit is a valuable tool for the measurement of membrane potential [78] and ion channel pharmacology [79,80]. Although electrophysiology is considered the gold standard for measurement of membrane potential, PMPI has several advantages, namely the ease of use, the ability to monitor long-term changes in multiple cells simultaneously. Furthermore, PMPI has been compared directly to electrophysiology data with a good agreement [68]. The quencher in the PMPI usually remains in the extracellular fluid due to its large size and continues to quench the fluorescence in the extracellular environment. However, when the plasma membrane is permeabilized, the quencher could potentially enter the cell through pores formed in the membrane and decrease the fluorescence in the cell. When we delivered pulses well above the depolarization threshold, we observed a decrease in a fluorescent signal which could be indicative of cell electroporation. In the future, it should be established to what extent the PMPI dye could also serve as cell membrane permeabilization indicator.

The results of electrochemical depolarization show that with increasing K^+ concentration, the fluorescence and thus the transmembrane voltage are increasing which is in agreement with theory. However, it was unexpected that the highest fluorescence was achieved with CHO cells and not with the differentiated HT22 cells. Excitable cells typically have a higher density of voltage-gated channels, and thus more ions should enter the cell when these channels are open. Results for electrical depolarization to 1 ms and 0.6 kV/cm show similar trends – the highest change in fluorescence was measured with the CHO cells, and the response of the differentiated and non-differentiated HT22 cells was similar (Fig. 5) which was surprising. CHO cells are non-excitable cells and would be expected to have a low background of voltage-

gated ion channels; however, some reports indicate that these cells express voltage-gated Na^+ channels [81] and a lack delayed rectifier K^+ currents [82]. This would explain why CHO cells exhibited more significant and prolonged depolarization in our experiments compared to other cells evaluated. Future investigations should examine the influence of voltage-gated Na^+ channel inhibitors on CHO depolarization. This finding highlights the importance of considering the endogenous complement of voltage-gated ion channels expressed in a given cell line. It is also commensurate with our recent results using PMPI voltage imaging to demonstrate the numerous ion channels present and implicated in the depolarization of U-87 MG cells by short electric pulses [83]. Another difference between the CHO cells and other three cell lines is their pattern of growth. CHO cells tend to form colonies and are in close contact to one another which causes electric field shielding and lower induced transmembrane voltage [84]. Cells of U-87 MG and HT22 cell lines were grown to be more isolated (Fig. 1).

Our strength-duration curve was determined for pulse lengths between 10 ns and 10 ms. As expected, the electric field needed to depolarize cells decreased with increasing pulse duration [71,85,86]. In [86], the strength-duration curve was measured for frog muscles between 1 ns and 100 ms. The authors determined that the thresholds for pulses of 100 μs or shorter followed a linear curve (in log-log scale) which confirmed that the signal was due to the opening of the ionic channels and not due to electroporation. Our results had roughly the same dependency but the linear curve followed a linear dynamics for pulses shorter than 1 μs . The differentiated HT22 cells are excitable [87] and because of similar shape of the strength-duration curve to the curve by Rogers et al. [86] we can assume that for short pulses we also measured predominantly opening of the voltage-gated channels and not membrane electroporation. The other three cell lines are not excitable, and the depolarization threshold only shows when the transmembrane voltage was significantly increased above the resting voltage. The reasons for the discrepancy between the data by Rogers et al. and our study could be that they used exponential pulses instead of square pulses used in our study, and determined the threshold by observing the twitching of the muscle while we used a fluorescent dye.

For the depolarization of the differentiated HT22 cells, higher electric field intensity was needed at most pulse lengths. These results are expected due to the more negative resting membrane voltage of differentiated non-dividing cells [88] for which a higher change in membrane potential is needed to reach the threshold of depolarization. However, at pulse lengths of 10 ns the difference in the critical membrane voltage between the differentiated HT22 cells and non-excitable cells was up to 0.6 V (approximating cells as prolate spheroids with $R_1 = 27 \mu\text{m}$ and $R_2 = 10 \mu\text{m}$), which

cannot be explained solely by a lower resting transmembrane voltage (around 50 mV difference between the excitable and non-excitable cells). The endogenous voltage-gated channels present in the plasma membrane of the CHO cells could explain this observation.

First, we tried modelling the depolarization data with Lapicque's curve which is similar to the Bleiss curve used in [86] but is valid for square pulses. We could not describe the shape of the strength-duration curve well, and the optimized value of the chronaxie was higher than predicted from the value of the rheobase. Thus, we decided to fit the Hodgkin-Huxley model, a more complex model, which takes into account the dynamics of the voltage-gated channels. The Hodgkin-Huxley model in [71] couples the voltage on the anodal and cathodal pole of the cell via the equivalent circuit. We modelled the cell as an idealized planar structure which does not accurately describe cell shape but it does give a general idea of the shape of strength-duration curve. Even when calculating the strength-duration curve for a spherical cell, the shape of the strength-duration curve remained the same, it only slightly moved along the y-axis (Fig. 7 in [71]). The shape of the strength-duration curve is not linear. The Hodgkin-Huxley model qualitatively followed the data well for pulses shorter than 10 μ s, but for longer pulses, the predicted depolarization threshold decreased more than the experimentally determined one. The reasons for deviation at long pulses could be the following. First, we were also electroporating cells enough for the dye to enter the cell but not the quencher. Second, our system was not sufficiently sensitive to determine very low depolarization thresholds. Third, for exact determination of the depolarization thresholds or action potential (for the differentiated HT22 cells) electrophysiological measurements are necessary. Fourth, also other channels contributed to the dependency of depolarization observed in our experiments but were not included in the model.

In [52] the authors achieved excitation of a peripheral nerve with a nanosecond pulse without electroporation. The presence of action potential without electroporation is in agreement with our modelling since the thresholds for depolarization were lower than electroporation thresholds. Additionally, the nerves' refractory properties were not affected.

We found the plateau of depolarization thresholds at 1 μ s intriguing and decided to try the same model on bipolar pulses. The results of this model offered an interesting perspective on the potential mechanism of the H-FIRE protocol [66]. In Fig. 6, we can see that around 1 μ s there is an overlap of the depolarization thresholds determined by the Hodgkin-Huxley model and of the Saulis permeabilization model. This overlap could explain why with 1 μ s bipolar pulses, electroporation was observed while muscle contractions were not [66]. In our model, with currently chosen parameters the threshold for electroporation was still higher than for an action potential, but the parameters of the Hodgkin-Huxley model were not optimized to describe our data, and we used the same values as in [71]. Optimizing the values of the Hodgkin-Huxley model could bring curves closer together. The Hodgkin-Huxley model and permeabilization model also indicate that short monopolar pulses could be better at not exciting the tissues since the threshold for action potential was calculated to be higher than that of the bipolar pulses. A similar explanation why the H-FIRE pulses do not excite the cells was offered in [89] by numerical modelling of the response of nerves to bipolar pulses. These authors showed that by using short bipolar pulses, it was possible to ablate a tissue region without triggering action potentials in the nearby nerve. The reason proposed being that the stimulation threshold rises faster than the irreversible electroporation threshold. Further experiments are now needed to test these hypotheses, comparing the thresholds for action potentials and electroporation in excitable tissues with monopolar versus bipolar pulses in the 1 μ s range.

The repolarization time of all four cell lines was in the range of minutes. The values for CHO, U-87 MG and non-differentiated HT22s are in agreement with the current knowledge existing as these cell lines do not have I_K voltage-gated potassium channels, responsible for fast

repolarization. The values of repolarization of differentiated HT22 cells are much longer than traditionally observed during neural depolarization, which is in the range of milliseconds. There are several possible explanations for this discrepancy. First, as the assessment method, we used PMPI dye entering the cell and then being pumped out. PMPI dye has a time constant of several seconds [90] and can be understood as a low-pass filter. Second, our experiments were performed at room temperature which slows down the speed of the Na/K pump. Third, it is possible that delivery of a single pulse led to a burst of action potentials and thus a prolonged depolarization. Fourth, that cells were depolarized as well as electroporated. Fifth, electroporation causes leakage of ATP [91] which is necessary for driving the pumps, and lack of ATP could slow them down. Sixth, due to high induced transmembrane voltage ion channels could be damaged [92,93].

The time required for reaching the peak fluorescence in depolarization experiments coincided with the resealing time observed in the permeabilization experiments. It is possible that during depolarization and permeabilization experiments, PMPI and YP were entering through the voltage-gated channels [94] as well as through pores formed in the plasma membrane. Even when using channel inhibitors, a total inhibition of depolarization could not be achieved which indicates that during depolarization ions also enter through pores [83].

An interesting study where also a fluorescent dye was used to assess depolarization/action potential and plasma membrane electroporation of hippocampal neurons was recently performed by Pakhomov et al. [95]. They determined that the activation of voltage-gated sodium channels enhanced the depolarizing effect of electroporation. The authors used the Fluo-Volt dye which enables imaging of fast changes in the range of ms. On the other hand, the PMPI dye enables imaging in the range of seconds to minutes, and it enables to capture slow, persistent changes in the transmembrane potential in the non-excitable cells.

In the next part of our study, we exposed cells to $8 \times 100 \mu$ s pulses, which are typically used in electrochemotherapy treatments. All four cell lines reached the threshold of electroporation at approximately the same value - between 0.4 and 0.6 kV/cm. The permeabilization curve of all four lines could be described using a symmetric sigmoid. Although the differentiation causes a drop in the resting membrane potential [88], the lower, i.e. more negative resting membrane potential did not affect the threshold of electroporation as it was similar for excitable and non-excitable cells. The permeabilization curves then followed similar dependency (Fig. 7b) and reached 50% of the maximal fluorescence around 0.9 kV/cm. We can conclude that irrespective of the excitability, all four cell lines responded similarly to electroporation pulses. The results are in agreement with electroporation being a physical means of disturbing plasma membrane in the lipid domain of the membrane. If the voltage-gated ion channels contributed to the YP uptake, it was much lower than the uptake through the permeabilized membrane.

The fluorescence reached was the highest for the U-87 MG cell line and the lowest for the CHO cell line while the fluorescence of the differentiated and undifferentiated HT22 cells was between the levels of the CHO and U-87 MG cells. Since the cells were grown attached in a monolayer, the lowest fluorescence of the CHO cell line can be explained by the tendency of CHO cells to grow in colonies in proximity which decreases the area of the plasma membrane available for dye uptake. Another reason could be that the proximity of cells decreases the induced transmembrane potential due to shielding [76,84,96] although it is unexpected that the proximity did not affect the threshold of electroporation. YP starts to emit fluorescent light after binding to nucleic acids. Thus different concentration of intracellular nucleic acids could also affect the fluorescence.

From the time-lapse images of the YP uptake, the resealing speed could be determined. The values of resealing constants were in the same range as in [77] although the electroporation buffer we used in this study was not tested previously. The cancerous U-87 MG cell line resealed much faster than the other (normal) three cell lines which is

in agreement with [97] where it was observed that cancerous cells resealed 2–3 times faster than normal ones due to lower tension levels in their lipid membranes.

6. Conclusions

In summary, the depolarization threshold was higher for the excitable cells than for the non-excitable cells. The strength-duration curve of excitable cells was described with the Lapicque curve and the Hodgkin-Huxley model. However, neither of the models described the behaviour at all pulse durations. The Hodgkin-Huxley model gave insight into the ability of the H-FIRE to permeabilize but not excite the tissue. All four cell lines responded similarly to pulses of standard electrochemotherapy parameters. The shape of the permeability curve was similar to curves already published in the literature [98]. Thus, electroporation is a feasible means of treating excitable and non-excitable cells with pulses of similar parameters. Furthermore, our results show the potential of achieving permeabilization and minimizing or avoiding excitation/pain sensation which needs to be explored in more detail. In future studies, it should be established, however, to what extent in vitro depolarization and excitability correlate to the actual excitation and pain sensation in vivo.

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Conflict of interests

The authors have declared that no competing interests exist.

Authorship

JDC: acquisition of the data, analysis and interpretation of the data, drafting the paper, final approval of the paper.

DM: conception and design of the study, analysis and interpretation of the data, drafting the paper, final approval of the paper.

MR: design of the ns-μs pulse generator, used in the study, final approval of the paper.

PM: design of the ns-μs pulse generator, used in the study, final approval of the paper.

SMB: acquisition of the data, analysis and interpretation of the data, final approval of the paper.

RB: acquisition of the data, analysis and interpretation of the data, final approval of the paper.

DAC: design of the ns pulse exposure setup, final approval of the paper.

PL: analysis and interpretation of the data, design of the ns pulse exposure setup, final approval of the paper.

ROC: conception and design of the study, acquisition of the data, analysis and interpretation of the data, drafting the paper, final approval of the paper.

Appendix A

Table A1

Statistical parameters of the strength-duration curve statistical analysis by ANOVA for the CHO cell line (Table A1a), the U-87 MG cell line (Table A1b) the undifferentiated HT22 (Table A1c) and the differentiated HT22 cell line (Table A1d). $F(x,y)$ means the F-test value, where x, y are the degrees of freedom of the between-group comparison and the within-group comparison. Where F_w is indicated, the assumption of homogeneity of variance was not met; therefore, a Welch's adjustment for the ANOVA was calculated and reported. The statistical significance provides the p-values which are compared to our alpha criterion of $\alpha = 0.05$. The effect size column provides an indication of the variability in response that can be attributed to the PEF. As an example, $\Omega^2 = 0.47$ means that 47% of the change in membrane potential can be attributed to the PEF.

CHO	F	p	Ω^2
10 ns	$F(2, 17) = 9.00$	$p < 0.01$	0.47
550 ns	$F(3, 15) = 4.56$	$p = 0.02$	0.36
1 μs	$F(3, 16) = 91.52$	$p < 0.01$	0.93
10 μs	$F_w(3, 6.08) = 14.12$	$p < 0.01$	0.67
100 μs	$F(4, 19) = 10.35$	$p < 0.01$	0.61
1 ms	$F_w(3, 5.55) = 53.76$	$p < 0.01$	0.89
10 ms	$F(3, 13) = 10.80$	$p < 0.01$	0.63

U-87 MG	F	p	Ω^2
10 ns	$F(4, 19) = 2.90$	$p < 0.01$	0.80
550 ns	$F(3, 15) = 3.60$	$p = 0.04$	0.29
1 μs	$F(3, 16) = 4.32$	$p = 0.02$	0.33
10 μs	$F_w(4, 9.27) = 43.54$	$p < 0.01$	0.63
100 μs	$F(3, 21) = 47.64$	$p < 0.01$	0.85
1 ms	$F_w(4, 6.62) = 13.96$	$p < 0.01$	0.71
10 ms	$F(3, 19) = 10.66$	$p < 0.01$	0.56

Undifferentiated HT22	F	p	Ω^2
10 ns	$F_w(3, 3.06) = 21.88$	$p = 0.01$	0.81
550 ns	$F(2, 9) = 12.96$	$p < 0.01$	0.67
1 μs	$F(3, 9) = 11.14$	$p < 0.01$	0.70
10 μs	$F(4, 10) = 5.24$	$p = 0.02$	0.53
100 μs	$F(2, 12) = 3.79$	$p = 0.05$	0.27
1 ms	$F(3, 10) = 9.06$	$p < 0.01$	0.63
10 ms	$F(4, 13) = 4.15$	$p = 0.02$	0.41

Differentiated HT22	F	p	Ω^2
10 ns	$F_w(3, 11.76) = 13.41$	$p < 0.01$	0.60
550 ns	$F(2, 10) = 5.20$	$p = 0.03$	0.39
1 μs	$F(2, 16) = 4.56$	$p = 0.03$	0.29
10 μs	$F(6, 21) = 3.97$	$p < 0.01$	0.39
100 μs	$F(4, 16) = 6.13$	$p < 0.01$	0.49
1 ms	$F(3, 19) = 8.80$	$p < 0.01$	0.50
10 ms	$F(4, 20) = 6.02$	$p < 0.01$	0.45

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