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The influence of Lidocaine on cell membrane permeabilization and cell survival after electroporation

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Abstract

Electroporation is a phenomenon in which exposure of a cell to pulsed electric field causes the formation of pores in the cell membrane. During the period of increased membrane permeability, otherwise impermeable molecules can be introduced into the cell or extracted from the cell. If the cell survives, this is called reversible electroporation; if the cell dies, this is considered irreversible electroporation. Both reversible and irreversible electroporation are used in different applications in medicine, including treatment of solid tumors. In these treatments, Lidocaine is often used as a local anesthetic to mitigate pain. However, a few studies also suggested that Lidocaine acts as a cell sensitizer for reversible and/or irreversible electroporation. To further study the potential of Lidocaine as a sensitizing agent, our investigation focused on assessing Lidocaine's effect on cell membrane permeabilization and cell survival in *in vitro* experiments, whereby 4 different cells lines (B16F1, C2C12, CHO and NS-HEK) were exposed to 8 x 100 µs pulses at repetition frequency of 1 Hz, as conventionally used for electrochemotherapy treatment. Our results suggest that 10 mM Lidocaine, dissolved in a physiological electroporation solution, has a minor effect on cell membrane permeabilization. However, a significant decrease in cell survival is observed, which can be attributed mainly to Lidocaine's inherent cytotoxicity.

1. Introduction

High-intensity pulsed electric fields are used increasingly in medicine [1] as well as in biotechnology [2] and food technology [3], to achieve a transient increase in the permeability of cell membranes. The applied electric field triggers a phenomenon called electroporation, which involves creation of hydrophilic pores in the lipid domains of cell membranes, oxidative lipid damage, and damage to certain membrane proteins [4]. By fine-tuning pulse parameters (duration, amplitude, number, repetition rate) we can achieve reversible (allows cell survival) or irreversible (leads to cell death) electroporation.

One important aspect of electroporation research are sensitization methods – methods that allow to increase the effects of electroporation at a given set of pulse parameters. This can for example be achieved by exposing the cells to hypotonic shock [5] or, in certain electroporation media, by splitting the applied pulse train (e.g., splitting 8 x 100 µs, 1 Hz pulses into two trains of 4 x 100 µs, 1 Hz pulses with 5 minutes interval in between) [6], [7]. Another potential method to facilitate membrane permeabilization and cell death is by using pharmacological agents to achieve cell sensitization. Specifically, Lidocaine was previously proposed as potential sensitizer [8], [9]. Lidocaine is an ion channel modulator, which primarily inhibits sodium voltage-gated channels and is used as a local anesthetic drug in clinical applications of electroporation, including electrochemotherapy (ECT). According to the standard operating procedures for ECT, Lidocaine can be used as a local anesthetic for the treatment of cutaneous tumors and skin metastases [10].

In this study we investigate how the presence of Lidocaine affects the increase in membrane permeability and cell survival when exposing cells *in vitro* to conventional ECT pulses (8 x 100 µs, 1 Hz) in physiological electroporation medium. The motivation for our study was based on two *in vitro* studies [8], [9] and one *in vivo* study [11] that reported a decrease in the threshold for reversible and irreversible electroporation in the presence of Lidocaine. However, the *in vitro* studies electroporated the cells in non-physiological low-conductive glucose solutions and used pulse parameters that are not conventionally used for ECT. The *in vivo* study was conducted on pig livers, where they observed larger legions of irreversibly electroporated cells in the presence of Lidocaine.

2. Methods

Cell lines

Experiments were performed using four different cell lines, Chinese hamster ovary cells (CHO-K1, #85051005), mouse C3H muscle myoblast (C2C12, #91031101) and mouse melanoma cells (B16F1, #92101203), all from the European Collection of Authenticated Cell Cultures. Additionally, we performed experiments on genetically modified human embryonic kidney cells that stably express voltage-gated sodium channels $Na_V1.5$, developed in the group of Adam E. Cohen at Harvard University [12], and now available from ATCC (cat. no. CRL-3479). While these cells can also express $K_{ir}2.1$ channels after incubating them with doxycycline, we used for our experimentsthe

variant of cells that express only $Na_V1.5$ (i.e., non-spiking, NS-HEK cells).

All cell lines were grown in humidified environment at 37 °C at 5% CO_2 and were routinely passaged every 3 to 4 days. Passages between 5-30 (and 3-15 for NS-HEK cells) were used for experiments. Each cell line was growth in its dedicated growth medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. The growth media were: Ham-F12 (Sigma-Aldrich, Germany, #N6658) for CHO-K1 cells, DMEM (Sigma-Aldrich, #D6546) for C2C12 cells, and DMEM (Sigma-Aldrich, #D5671) for B16F1 and NS-HEK cells.

For experiments, cells were first trypsinized and counted. Afterwards, cells were centrifuged for 5 minutes/0.2 RCF and the pellet was then resuspended in Tyrode buffer (composition described in Section Electroporation buffer) to obtain a final cell density of $1x10^6$ cells/ml.

Electroporation buffer
Tyrode buffer was used as the electroporation medium for all cell lines. Buffer was prepared in our laboratory in final composition of 125 mM NaCl (Sigma-Aldrich, Germany, #SI-71382), 2 mM KCl (Merck, Germany, #1049360550), 2 mM $CaCl_2$ (Sigma-Aldrich, Germany, #SL-C4901), 1 mM $MgCl_2$ (Sigma-Aldrich, Germany, #M8266), 10 mM HEPES (Merck, Germany, #1101100250), and 30 mM Glucose (Merck, Germany, #MC-1083371000). The conductivity of the prepared buffer was 14.66 mS/cm (at 24.7˚C), measured with conductometer SevenCompact (Metler Toledo). Tyrode buffer pH was titrated to 7.3 using NaOH (Merck, Germany, #1.06498.1000).

To assess the influence of Lidocaine on membrane permeabilization and/or cell survival Lidocaine HCl (prepared by the Pharmacy of the University Medical Centre Ljubljana, Slovenia; 20 mg/ml = 2% solution) was added to the cells, after they were resuspended in the Tyrode buffer, in final concentration of 10 mM. The cells were incubated in presence of Lidocaine for 10 minutes before exposing them to electric pulses. The concentration and incubation period were determined based on a previous *in vitro* study [8].

Cell permeabilization
Cell suspension (150 µl, $1x10^6$ cells/ml in Tyrode buffer with or without 10 mM Lidocaine) was mixed with propidium iodide (PI, Molecular probes, #P1304MP) in a final concentration of 100 µg/ml. The suspension was placed in electroporation cuvettes with 2 mm gap distance and exposed to 8 x 100 µs pulses of different amplitudes at repetition frequency of 1 Hz using a prototype pulse generator (L-POR V0.1, mPOR, Slovenia). The electric field strength to which the cells were exposed was estimated as the ratio between the applied voltage and electrode distance. PI is a membrane-impermeable fluorescent agent, commonly used in methods to assess the electropermeabilization of cell membranes [13]. 3 minutes after pulse application, 350 µl growth medium was added to the cell suspension and the sample was removed from the electroporation cuvette. The emission of PI fluorescence in the sample was detected by flow cytometry (Attune NxT, Carlsbad, CA, USA) using blue laser excitation at 488 nm and detecting the emitted fluorescence through a 574/26 nm band-pass filter. 10,000 events were obtained, and data were analyzed using the Attune Nxt software. Fluorescence intensity histograms were used to determine the percentage of PI permeabilized cells. Gating was set according to sham control (0 V, without Lidocaine). Measurements for each data point were repeated three times on three different days.

Cell survival
Cell suspension (150 µl, $1x10^6$ cells/ml in Tyrode buffer with or without 10 mM Lidocaine) was transferred into electroporation cuvettes, and electric pulses were applied in the same way as for cell permeabilization experiments. After pulse application, we waited for 10 minutes and added 850 µl (CHO-K1, C2C12, B16F1 cells) or 350 µl (NS-HEK) of growth medium supplemented with 10 mM HEPES solution BioXtra 1M (Sigma-Aldrich, Germany, #SI-H0887). A lower volume was used for NS-HEK cells, since we needed to plate a higher number of cells due to their lower growth rate. Afterwards, 100 µL of suspension with 1.5×10^4 cells/mL (for B16F1, C2C12 and CHO cells) or 3×10^4 cells/mL (for NS-HEK cells) was transferred from electroporation cuvettes in triplicates into a 96-well plate (TPP, Switzerland) and incubated at 37 °C and humidified 5% CO_2 atmosphere. MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA), which is a colorimetric method for determining the number of viable cells [13], was used to assess cell viability 24 hours after electric pulses were applied. According to the manufacturer's instructions, 20 µl of MTS tetrazolium compound was added to the samples, and the 96-well plate was returned to the incubator for 2 hours. The absorbance of formazan (reduced MTS tetrazolium compound) was measured with a spectrofluorometer (Tecan Infinite M200, Tecan, Austria) at 490 nm. The percentage of viable cells was calculated by first subtracting the background (signal from blank wells containing growth medium and MTS only) from all measurements and then normalizing the absorbance of a given sample to the absorbance of the sham control samples. Measurements for each data point were repeated three times on three different days.

Statistical analysis
Statistical analysis was performed using Student's t-test in SigmaPlot 11.0 (Systat Software, USA). All results are presented as mean ± standard deviation (SD) of three independent experiments.

3. Results

We first assessed the influence of Lidocaine on the increase in cell membrane permeability after exposing cells to conventional ECT pulses (8 x 100 µs) of different amplitudes. Results presented in Fig. 1 show that the percentage of permeabilized cells (cells stained with PI)

increases with increasing pulse amplitude, consistent with previous studies [14]. The presence of 10 mM Lidocaine did not significantly affect cell permeabilization in CHO, B16-F1 and NS-HEK cells. However, there was a significant increase of ~20% in the percentage of permeabilized C2C12 cells at 1 kV/cm. Therefore, Lidocaine potentiated cell permeabilization to a small extent, but this effect was significant only in one type of cells.

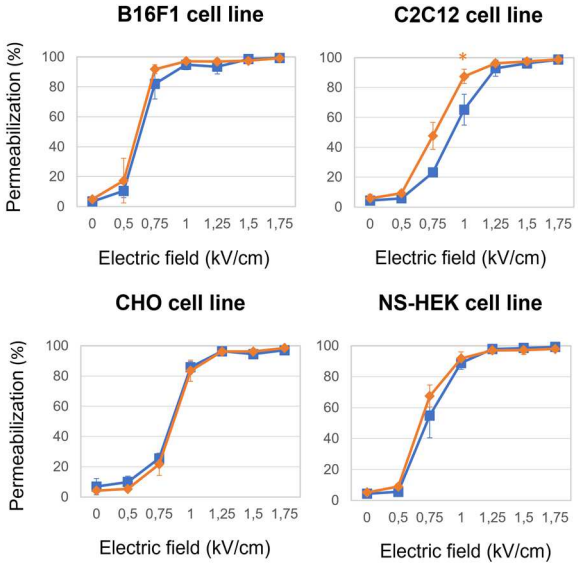

Figure 1. Percentage of permeabilized cells (♦ with 10 mM Lidocaine added; ■ without Lidocaine added), depending on the electric field strength. The results are shown as mean ± SD, N=3. Statistically significant differences (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) were determined by t-test.

We next assessed the ability of Lidocaine to potentiate cell death after exposure of cells to the same pulse parameters (8 x 100 µs, 1 Hz) as for cell permeabilization experiments. The results presented in Fig. 2 demonstrate that Lidocaine significantly decreased cell survival in all tested cell lines (cf. ■ and ●). However, this effect can be mainly attributed to Lidocaine's inherent cytotoxicity – note that significant decrease in cell survival was already observed in the absence of applied electric pulses (at 0 V/cm). The cytotoxic effect of Lidocaine varied between different cell types and was most profound in B16-F1 and NS-HEK cells. To determine whether there was any synergistic effect between cell death due to Lidocaine and irreversible electroporation, we re-plotted the results for cell survival with Lidocaine; instead of normalizing the values to the sham control without Lidocaine (●), we normalized the values to the sham control with Lidocaine (♦). Using this approach for presenting the results, significantly lower cell survival was observed only in B16F1 and NS-HEK cells at 1 kV/cm, and in CHO cells at 1.5 kV/cm. Interestingly, there was no significant difference between survival curves in C2C12 cells (cf. ■ and ♦), even though this cell line was the only one to exhibit significant increase in cell permeabilization with Lidocaine (Fig. 1).

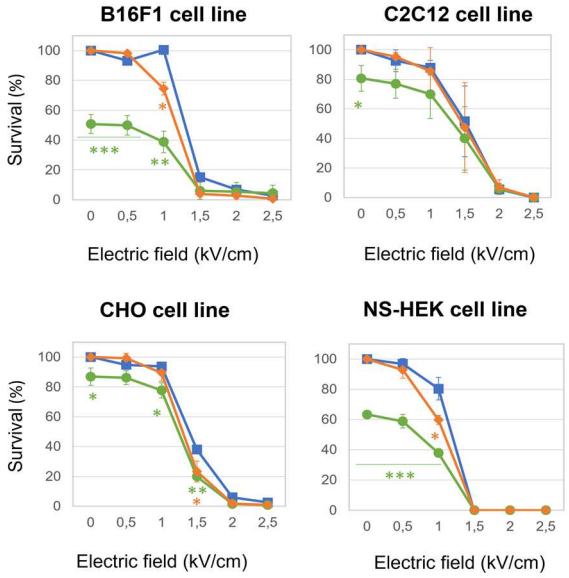

Figure 2. Percentage of survived cells (♦ with 10 mM Lidocaine added, normalized to control with Lidocaine; ● with 10 mM Lidocaine added, normalized to control without Lidocaine added; ■ without Lidocaine added). Residual of Lidocaine in the growth medium for determining cell survival with MTS after 24h: 1.5 mM (for B16F1, C2C12 and CHO cells) and 5 mM (for OS-HEK cells). The results are shown as mean ± SD, N=3. Statistically significant differences (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) were determined by t-test.

4. Discussion

Our results suggest that Lidocaine can sensitize cells to reversible and irreversible electroporation, but to a small extent and in a cell type dependent manner. Overall, contrary to previous reports [8], [9] we did not observe a major potentiation of reversible and irreversible electroporation in the presence of Lidocaine. In our study we electroporated the cells in Tyrode buffer with physiological conductivity and pH, whereas in both previous studies [8], [9], cells were electroporated in low-conductive media with no reported pH value. Lidocaine has a pKa value of around 8, meaning that already a small deviation from physiological pH value can significantly affect the form of Lidocaine (protonated vs. deprotonated). Molecular dynamics simulations have shown that protonated and deprotonated form of Lidocaine interact with lipid membrane in a different way; protonated form prefers to reside at the membrane interface, whereas deprotonated form can penetrate deep into the middle of the bilayer [15]. Protonated and deprotonated forms of Lidocaine could potentially affect membrane electroporation differently. Similarly, the conductivity of the medium could influence the interaction of Lidocaine with the cell membrane, since low-conductive buffers with

lower ionic strength have lesser ability to screen the negative charge on the cell membrane and positive charge of Lidocaine in protonated form. Therefore, further studies investigating the role of medium pH and conductivity on Lidocaine's ability to influence electroporation are required.

Another important aspect that we observed in our study is Lidocaine's inherent cytotoxicity. It should be noted that following electroporation, cells were grown for 24 h in the presence of at least 1.5 mM residual Lidocaine. Therefore, protocol should be optimized by removing the Lidocaine before plaiting the cells for evaluation of cell survival.

4. Conclusions

Lidocaine is commonly used as a local anesthetic in electroporation-based medical treatments such as electrochemotherapy and it was proposed as cell sensitizer for reversible and/or irreversible electroporation. Our findings indicate that when cells are electroporated in the presence of Lidocaine in physiological solution, Lidocaine does not potentiate membrane permeabilization, but it can influence cell survival in all tested cell lines. However, it is important to note that the reduction in cell survival is mainly due to Lidocaine's intrinsic cytotoxicity, and we observed very limited synergistic effect between Lidocaine and cell death due to irreversible electroporation. Further experiments are needed to understand the role of electroporation medium composition on Lidocaine's ability to act as a sensitizer.

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