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# Transient suppression of gap junctional intercellular communication after exposure to 100-nanosecond pulsed electric fields

Anna Steuer [a], Anke Schmidt [a], Petra Labohá [a,b], Pavel Babica [b], Juergen F. Kolb [a,*]

[a] Leibniz Institute for Plasma Science and Technology, Greifswald, Germany
[b] Research Centre for Toxic Compounds in the Environment (RECETOX), Faculty of Science, Masaryk University, Brno, Czech Republic

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ABSTRACT

Gap junctional intercellular communication (GJIC) is an important mechanism that is involved and affected in many diseases and injuries. So far, the effect of nanosecond pulsed electric fields (nsPEFs) on the communication between cells was not investigated. An *in vitro* approach is presented with rat liver epithelial WB-F344 cells grown and exposed in a monolayer. In order to observe sub-lethal effects, cells were exposed to pulsed electric fields with a duration of 100 ns and amplitudes between 10 and 20 kV/cm. GJIC strongly decreased within 15 min after treatment but recovered within 24 h. Gene expression of Cx43 was significantly decreased and associated with a reduced total amount of Cx43 protein. In addition, MAP kinases p38 and Erk1/2, involved in Cx43 phosphorylation, were activated and Cx43 became hyperphosphorylated. Immunofluorescent staining of Cx43 displayed the disassembly of gap junctions. Further, a reorganization of the actin cytoskeleton was observed whereas tight junction protein ZO-1 was not significantly affected. All effects were field- and time-dependent and most pronounced within 30 to 60 min after treatment. A better understanding of a possible manipulation of GJIC by nsPEFs might eventually offer a possibility to develop and improve treatments.



## 1. Introduction

Pulsed electric fields applied to living cells can induce different biological effects depending on pulse duration and field strength. Pulses in the range of milli- to microseconds primarily affect the outer membrane, while shorter pulses with duration in the nanosecond range have a more pronounced effect on subcellular structures. Interest in the study of pulsed electric field exposures arises mainly from the potential to induce different biological responses which can be exploited for medical applications. Among those successfully in use in the hospital already are electrochemotherapy by using reversible electroporation [1–5] and tissue ablation by irreversible electroporation [6,7]. Both methods use rather long electrical pulses of microsecond or even millisecond duration. Pulsed electric field exposures with individual pulse durations that are short compared to the charging time of the outer membrane, i.e. nanosecond pulsed electric field (nsPEF) exposures, have been found to induce apoptosis in cancer cells and are currently investigated with respect to cancer treatment [8–13].

The development of medical therapies is associated with the need of a deeper understanding of interactions between pulsed electric fields and cells. Although the fundamental and direct mechanism must be an effect on charges and dipoles of cellular constituents, subsequent biochemical responses and their complex relationship are deciding on the fate of a cell. A distinction between first or direct physical mechanisms and induced secondary or indirect biochemical processes is sought after to establish a chain of events but often difficult to establish [14,15]. Investigations on the mechanisms of the interaction of pulsed electric fields with cells were so far mostly done on single cells. Some of the studied effects are for example the formation of pores in the plasma membrane, the release of calcium from intracellular stores or the breakdown of the cytoskeleton [14,16–21]. Conversely, in animal studies in particular the possibility for cancer treatments was intensively investigated [10,12,22]. However, between the study of single cells and the complex responses of tumors in an animal, only little is known about the direct effects of nsPEF on cells that are connected and communicate with each other in a tissue.

Gap junctional intercellular communication (GJIC) is important for the regulation of cell growth control, differentiation and apoptosis. An impaired cell-cell communication is a crucial factor in many diseases such as skin diseases, deafness and cataract [23,24]. Furthermore, it plays an important role in cancer and metastasis formation as well as in wound healing [25–35]. Direct communication between mammalian cells is mediated by gap junctions. These are water-filled pores that directly connect adjacent cells. One connexon (hemichannel) consists of six connexin (Cx) proteins and two connexons form one gap junction (GJ). 21 members of the Cx-gene family are known in humans and 20 in mice, with the

* Corresponding author at: Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Strasse 2, 17489 Greifswald, Germany.
*E-mail address:* juergen.kolb@inp-greifswald.de (J.F. Kolb).

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encoded proteins named after their molecular weight, e.g. Cx43. GJs allow the diffusion of ions or signaling molecules with a size up to 1 kDa to adjacent cells. Cell-cell communication is not only regulated by the amount of GJs in the membrane but also by their opening state (gating). The opening state is influenced by different factors such as Cx phosphorylation, membrane polarization and intracellular pH-value and calcium level [36,37]. Cx phosphorylation by mitogen-activated protein (MAP) kinases including extracellular signal regulated kinases (Erk), p38 and C-Jun N-terminal kinases (Jnk) represents probably the most characterized and studied mechanism of GJIC regulation [38–40].

NsPEFs applied to cells can induce the breakdown of the cytoskeleton [20,41,42]. An intact actin and microtubule cytoskeleton is necessary for building and degradation of GJs. After the chemically induced disruption of the cytoskeleton connexin proteins accumulate in the cytoplasm while the amount of membrane-bound connexin decreases. Thus, inhibition of the cytoskeleton is expected to affect GJIC either directly by influencing the mechanisms of the channel transfer or indirectly by disturbing the correct assembly of the GJs [43].

For the study of nsPEF-induced effects on GJIC and the mechanisms involved in its regulation, the rat liver epithelial cells WB-F344 [44] were grown and exposed in monolayers. These non-cancer, stem-like cells have a high expression of Cx43 and, unlike most other cell lines, communicate strongly via GJs in vitro. This cell line has been widely used as a model system for the study of gap junctional intercellular communication by Trosko et al. [45–49].

The aim of our experiments was to investigate the effects of sub-lethal exposure conditions. Therefore, we chose a pulse length of 100 ns that has often been used in animal studies [11,13,50,51] and rather mild field strengths of ≤ 35 kV/cm. After the application of trains of 20 pulses to confluent monolayers, GJIC and related parameters and markers were investigated, including gene and protein expression of Cx43 and activation of different MAP kinases. In addition, Cx43 distribution and actin cytoskeleton rearrangements that are directly related to ability of cells to communicate were determined.

## 2. Materials and methods

### 2.1. Cell culture

WB-F344 cell line was derived from normal adult male Fischer 344 rat liver by J.W. Grisham and M.S. Tsao of the University of North Carolina at Chapel Hill, Chapel Hill, NC, USA [44], and the culture was obtained from Prof. J. E. Trosko, Michigan State University, East Lansing, MI, USA. WB-F344 cells are diploid, non-tumorigenic, possessing characteristics of normal liver progenitor or bipolar stem cells, whose intrahepatic transplantation into adult syngenic F344 rats results in the morphologic differentiation of these cells into hepatocytes and incorporation into hepatic plates [52]. WB cells express Cx43 and have been extensively characterized for alterations of GJIC in the absence and presence of well-known tumor promoters, growth factors, tumor suppressor genes, and oncogenes [28].

WB-F344 cells were cultured in low-glucose DMEM (#8950614), supplemented with 2 mM L-glutamine, 5% fetal calf serum and 1% penicillin/streptomycin (#4211014) (all purchased from PAN-Biotech GmbH, Aidenbach, Germany) in a humidified $CO_2$ (5%) incubator. Cells were seeded in 12-well plates. Exposures were done after 48 h of cell growth, when a confluent monolayer was achieved.

### 2.2. Experimental setup

The experimental setup (Fig. 1) for the application of electric pulses to a monolayer consists of the in-house developed pulse generator, the power supply (FX20R15, Glassman, High Bridge, NJ), a fast oscilloscope (TDS3054, Tektronix, Beaverton, OR) and a high voltage probe (P5100A, Tektronix). The pulse-generator is based on the Blumlein concept and delivers pulses with a duration of 100 ns [53]. The electrode

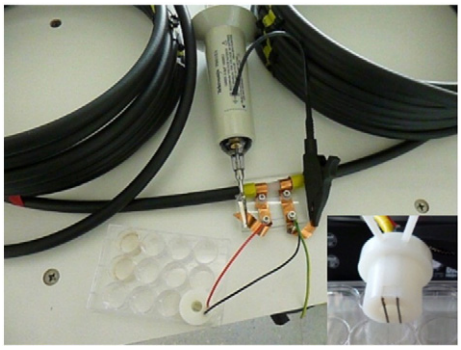

**Fig. 1.** Overview of the experimental setup with pulse generator, electrode configuration and high voltage probe. Electrodes are embedded in a nylon-cylinder to apply the electric pulses to a monolayer in a 12-well plate.

configuration consists of two parallel stainless steel wires fixed in a plastic cylinder that was tightly fitting in the well of a 12-well plate. The wires have a diameter of 0.8 mm and a gap distance of 5 mm measured from the center of each electrode. The electric field was applied by slightly impressing the electrodes into the monolayer until the electrodes touched the bottom of the well. 20 pulses with amplitudes between 5 and 35 kV/cm were applied.

### 2.3. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added within 2–10 min after treatment to analyze the respiratory activity of the cells which correlates with cell viability. When a confluent monolayer was achieved cells were exposed to nsPEFs in cell culture medium. Then, cell culture medium was replaced by 500 μl medium and 50 μl MTT-solution (5 mg/ml in PBS; AppliChem, Darmstadt, Germany). After 2 h of incubation at 37 °C the MTT-solution was removed and the monolayers were washed once with HBSS. The cells were then lysed with a cell lysing buffer (99.4 ml DMSO, 0.6 ml acetic acid (100%), 10 g SDS). After 5 min incubation time and 5 additional minutes on a shaker the absorbance of the lysate was measured at 550 nm with a reference wavelength of 700 nm (Infinite M200 PRO, Tecan, Männedorf, CH). In each experiment, the weighted absorption values were determined in triplicates for each set of exposure parameters.

### 2.4. Scrape loading/dye transfer assay

Scrape loading/dye transfer (SLDT) assay was performed to investigate cell-cell communication [54]. After a certain incubation period of the nsPEF treated cell monolayers medium was removed and replaced by 0.05% Lucifer Yellow (L0259, Sigma-Aldrich, Taufkirchen, Germany) and 0.1% dextran conjugated Texas Red (10,000 MW, D-1828, Invitrogen, Darmstadt, Germany) dissolved in PBS. A razor blade was used to make a scrape across the treated cells which were then incubated for 2 min to allow dye uptake by the damaged cells along the scratch. If the cells are connected by functional GJs, the GJ-permeable Lucifer Yellow (LY) diffuses to adjacent cells whereas the GJ-impermeable Texas Red-Dextran (TR-Dx), which serves as loading control and to exclude dye uptake due to electroporation, remains only in the damaged cells. Afterwards, cells were rinsed with PBS and fixed with 4% paraformaldehyde. Finally, monolayers were observed under a fluorescent microscope (Axio Observer D1, Carl Zeiss, Berlin, Germany).

The fluorescent area of the LY migration from the scrape line was quantified by using MATLAB. For this purpose, fluorescence images were converted into black-and-white-images by defining a threshold-value which determined the brightness at which pixels were converted into black and white, respectively. This threshold was kept the same for the evaluation of each experiment. The fluorescent areas of the LY channel of the treated cells were then normalized to the areas of the control