ASYMMETRIC CHANGES IN MEMBRANE CONDUCTANCE DUE TO HYPER- AND DEPOLARIZATION: PROBING WITH CURRENT AND VOLTAGE CLAMP

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Abstract: Cell membranes are composed differently at the cytosolic and the extracellular side. This has a strong effect on the current-voltage-characteristics (CVC) even if voltage gated channels are ignored. While a decrease of the conductance for small superimposed transmembrane voltages (<0.5 V) was found when chinese hamster ovary cells (CHO)–cells with no excitable plasma membrane hyperpolarized, the conductance rose as soon as a depolarizing voltage was applied. At higher voltage, both hyper- and depolarized membranes showed electroporation, but at different thresholds. Probing the CVC with controlled current or controlled voltage yields quite different results because of the variable voltage divider between the membrane and the electrolytes, which yields a positive feedback for voltage clamp condition but a negative feedback under current clamp condition. This also influences the results of pulsed field experiments in low (negative feedback) and highly conductive (positive feedback) media, indicating, for instance, a lower electroporation threshold of cells in tissues.

Introduction

The asymmetry in the electrical response of membranes at anodic and cathodic sides of cells in an imposed electric field was first observed by Hibino and coworkers [1]. Their explanation was that the superimposed electric field from the resting transmembrane potential increases the field strength at the anodic side while weakening it at the cathodic side. Thus, electroporation conditions are reached first at the anodic side.

Under physiological conditions, the interior of a cell is negatively charged with respect to the surrounding medium. The transmembrane potential difference of a resting cell is usually between -50 and -90 mV, and depends on the cell type, the surrounding medium and the physiological state. A stimulus, lowering the potential inside the cell hyperpolarizes the membrane, while any increase of the intracellular potential causes depolarization. Even if the cell membrane is recharged with reversed polarity, it is still considered depolarization.

Using whole-cell-clamp conditions, it is possible to obtain hyper- and depolarization selectively at cell membranes. Current or voltage-clamp measurements provide quite different results in the sense that a current-clamp appears less destructive to membrane structures because the field strength increases only slightly after a critical level is reached (voltage regulator effect) [2]. In the case of a voltage clamp, the potential difference at the membrane is clamped, and thus, if the conductance of the membrane increases, a current rise results with no regulatory feedback, i.e. the electric field causing the conductance increase remains unaffected.

Consequently, the conductivity of the suspension medium has a great impact in electroporation studies. A high conductivity corresponds to voltage clamp, while a low conductivity provides current clamp. It implies that with the same field strength applied, the effect of electroporation is higher in a highly conductive medium.

Material and Methods

Whole cell clamp Glass capillaries (PG120T-7.5 HARVARD Part No. 30-0091) as well as borosilicate glass capillaries (GC120T-7.5 HARVARD Part No. 30-0049) have been used to clamp the cell (Fig.1). They have been pulled to a diameter between 1 and 3 μm using a DMZ-Universal Microelectrode Puller (Zeitz, Munich, Germany). The pipettes have been filled either with 140 mM KCl or culture medium. In both cases, the pipette had a resistance between 2 and 3.5 MΩ.

The pipette was fixed at one wall of the acrylic measurement chamber and had an incorporated Ag/AgCl – electrode. The reservoir of the chamber was filled with culture medium. A liquid flow into the pipette was initiated by carefully reducing the pressure. Once the cell attaches to the tip, an electrical connection between cytosol and the electrode is established by breaking the cell membrane inside the pipette. The procedure of cell attachment was monitored by video microscopy (63 x, phase contrast, water immersion objective) and verified by impedance measurement of the cell membrane. Since the chamber

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is not temperature controlled, the experiments are conducted at room temperature.

Figure 1: CHO-cell sucked onto a capillary. The diameter of the cell is 14 μm.

The counter electrode (Ag/AgCl in 3 M KCL-agar), was in contact with the surrounding medium. An electric source (Fig.2) (voltage or current source) is controlled by a microcontroller-based arbitrary function generator. Current or voltage control is determined by getting feedback from either the current through the pipette or the voltage across the membrane. The membrane voltage is acquired at the output of a compensation circuit, subtracting the pipette voltage from the voltage measured across the pipette and cell membrane. This compensation has to be adjusted before each experiment. In order to compensate for the pipette voltage under current clamp conditions, an identical measurement is made for the pipette without the cell attached. Since the current density is the same, the voltage across the pipette can be subtracted yielding only the membrane voltage. The offset arising from the equipment is adjusted to zero while the liquid junction potentials were corrected using the generalized Henderson equation.

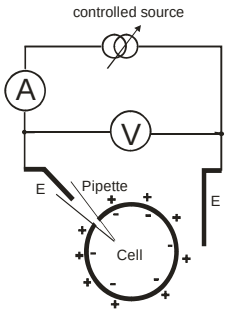

Figure 2: Schematic for the electrical connection of the cell.

Arbitrary function generator This device generates any waveform with a temporal resolution of 500 μs for 10 s. A higher time resolution was possible by decreasing the total time, or vice versa, since the microcontroller had only 64 kB of memory. The bandwidth of the entire system including the pipette is on the order of 10 kHz. Depending on the feedback, either the voltage across the membrane or the total current is controlled with 12 bit resolution. The applied voltage is in the range $-10 \le U/V \le +10$; the current is controlled in the range $-350 \le I/nA \le +350$. Decreasing the feedback gain yields a higher resolution, but lower output swing. Both the total current and the voltage across the pipette are monitored with 12 bit resolution. The head stage with the matching amplifier is mounted onto the microscope which has been placed in a Faraday cage for electric shielding. The basic unit is a desktop device, connected to a PC via RS232. The microscope with the head stage is mounted on a granite plate for acoustic damping. While the PC-programming was done in C++ for the driver and MATLAB for the user interface, the device itself is programmed in ASSEMBLER for efficient program execution. The highly automated processing of the video sequences is programmed in MATLAB.

Pulse protocols. Depending on the aim of the experiment, we adjust the pulse shape. For instance, the CVC of the plasma membrane of a single cell is derived from the response to ramp functions. Rectangular pulses are applied when we study the relaxation kinetic parameters at a constant membrane field. Prior to, and immediately after the stimulus, we apply a rectangular wave with a repetition frequency of 100 Hz with low amplitude (i.e. ± 3 nA) in order to assess the impedance of the membrane by time domain spectroscopy. The measurements before and after the electrical stimulus also yielded the trans-membrane voltage (current clamp) or the short circuit current of the membrane (voltage clamp).

Cell preparation Chinese Hamster Ovary cells (CHO) are used throughout our experiments. The culture medium (DMEM/F12) contained 5% FCS as well as 10-100 mg/L antibiotics like benzyl penicillin and streptomycin sulphate. The incubator was maintained at 37°C (310 K) and a CO_2-level of 5%. Since we conduct single cell measurements, they are highly diluted to prevent cell-cell interactions. CHO cells do not have excitable membranes, thus no precaution is taken to block voltage gated Na^+ and K^+ channels.

Video microscopy All experiments have been monitored by a video equipped microscope. The video-stream has been stored, together with the horizontal synchronization signal and the electrical stimulus using the audio channels (left, right) of the computer. The use of the synchronization signal made it simple to process the frames automatically using MATLAB. Each cell was examined at the end of the pulse for negligible changes during the electrical stimulus. If the preset value of discrepancy was exceeded, the experiment was marked as suspicious and reexamined visually frame-by-frame. In most cases, this revealed a cell detached from the pipette or a cell heavily damaged due to destructive pulsing conditions. It should be noted here that objectivity was assured by storing all results, independent of the quality of the experiment.

Results

Current and Voltage Clamp In general, passive electrical behavior, like the current-voltage relationship, is determined by application of an electrical stimulus and measurement of the system

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response. The stimulus can be a voltage or a current while the response is the current or the voltage respectively. For linear and time invariant systems, each measurement procedure yields the same result. However, non-linear systems, like the plasma membrane of cells, show different behaviors, depending on the stimulus applied. Fig. 3 shows the response of a CHO – plasma membrane to a current stimulus. The membrane is electrically stable up to a voltage of about 200 mV. A current of ± 50 nA does not cause it to exceed this limit and is, thereby, not dramatically changing the dielectric behavior of the membrane. While some voltage-gated channels exist, even within the non excitable CHO-plasma membrane, they do not dominate the electrical response of the membrane. The membrane is charged during the first 200 to 300 µs, limited by the conductance of the membrane. Increasing the charging current will increase the voltage. When a critical voltage is reached (i.e. 0.72 V for depolarization and 0.93 V for hyper-polarization in Fig.3), membrane electroporation (MEP) occurs. Since this lowers the membrane resistance, the trans-membrane voltage drops due to the constant current.

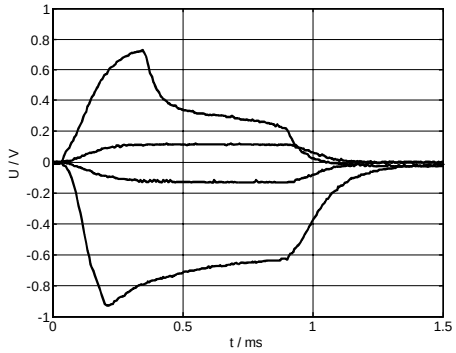

Figure 3: Transmembrane voltage due to a 1 ms current (from top: 250 nA, 50 nA, - 50 nA, -250 nA)

If the voltage across the membrane is held constant after the membrane is charged, the current continues to rise (Fig.4.). The marked difference between current and voltage clamp is the feedback. While MEP during current clamp decreases the electric field within the membrane dramatically and thereby reduces the probability of additional pore creation, the field is unchanged during voltage clamp. With respect to possible changes on the membrane, current clamp exhibits a negative feedback. While the membrane field does not change during voltage clamp, Joule heating can increase the probability of electroporation, providing positive feedback. The current increase during a constant voltage stimulus reflects the permeability increase of the membrane which, for a trans-membrane voltage below 1 V, is assumed to be due to the creation of aqueous pores spanning the lipid portion of the membrane.

Hyper- and depolarization The CVC of membranes is measured using ramp functions, either voltage- or current-controlled. A positive stimulus first depolarizes the membrane and later re-polarizes it

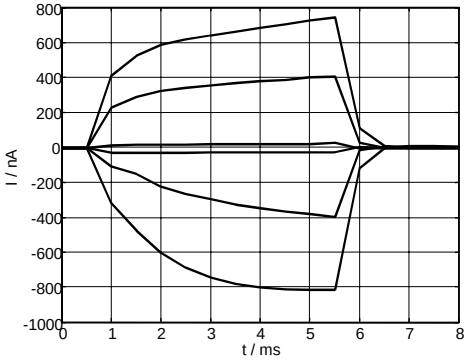

Figure 4: Transmembrane current due to the application of a constant voltage across the cell membrane. While a voltage jump across the capacitive membrane is not possible, the charging time under voltage clamp condition is on the order of 10 µs, much less than the time-scale shown for current increase in the figure. (from top: 800, 600, 200, -200, -600 - 800 mV).

with the opposite polarity. A remarkable difference is the faster increase of the membrane conductance at the depolarization side (Fig.5). Especially during current clamp experiments, we usually found a voltage regulator effect in the cell membrane, which kept the transmembrane voltage typically in a region between 300 and 600 mV. Under both, voltage- and current-clamp conditions, a lower increase in conductance occurs during hyper-polarization. This allows the cell to reach a higher transmembrane voltage without membrane rupture. A striking result is the decline in conductance associated with the current-controlled hyperpolarization.

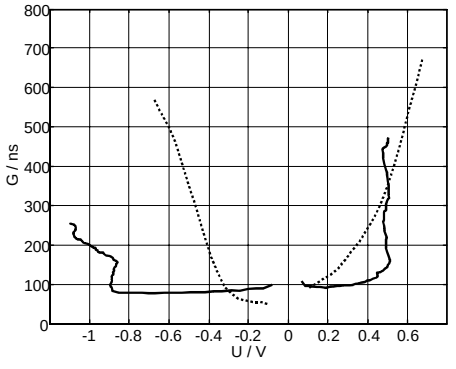

Figure 5: Membrane conductance depending on the trans-membrane voltage for comparable current and voltage clamp condition. Solid line: current clamped ramp (± 300 nA, 10 ms), dashed line: voltage clamped ramp (± 700 mV, 10 ms). The selected data show typical behavior and are chosen from a large number of experiments (about 60 interpretable experiments for these settings).

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A more general view, using the averaged results for quite different conditions, as would be found in tissue or inhomogeneous cell suspensions (i.e. high concentration), is given in Fig. 6. Because the individual levels for voltage regulation are different, the clear voltage regulation appearing in current-controlled experiments is absent. Moreover, the difference for depolarization in current- and voltage-clamp conditions declines.

In contrast, at hyperpolarization, almost no change in conductance was found up to 600 mV. But individual experiments, especially with slow rising ramps, already show a conductance change at much lower voltage.

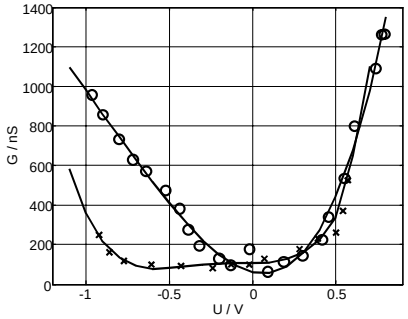

Figure 6: Membrane conductance during hyper- and depolarization averaged over all experiments (≈ 300) fulfilling the requirements for interpretation: (1) healthy looking cell, (2) cell was attached, (3) impedance before electrical stimulus was higher than 50 Ωcm^2 (4) every piece of equipment was working. The data averaged here are obtained by different persons and include different ramp rise time (5 ms .. 1s), voltage (200 mV .. 2 V, open circles) and current (50 .. 350 nA, crosses). For long-lasting stimulus, only small amplitudes are possible. Maintaining a voltage of more than 1 V is usually not possible, since the current rises to values which exceed the capabilities of the equipment (1 μA). Although a small change in the equipment would solve this limitation, the cell usually falls apart (vesicle formation) under these conditions. The values between -30 mV and 30 mV are interpolated, since the calculation of the conductance with almost zero current and alternating polarity (noise) becomes questionable.

Current/voltage characteristics depending on the slew rate Since structural changes on the membrane level take time (Fig.3 and 4), a slowly rising ramp has a stronger effect at the same transmembrane voltage. As seen in Fig.7, with a lower slew rate, a higher conductance was achieved at lower voltage. While nothing unexpected was found during depolarization, saturation was found for hyper-polarization. In general, higher voltages without destruction of the cell was achieved during hyperpolarization.

Long lasting stimuli If a membrane is considered to be homogeneous, composed of the same lipids at the cytosolic and the extra-cellular side, one would not expect different behavior of the membrane during de- or hyperpolarization.

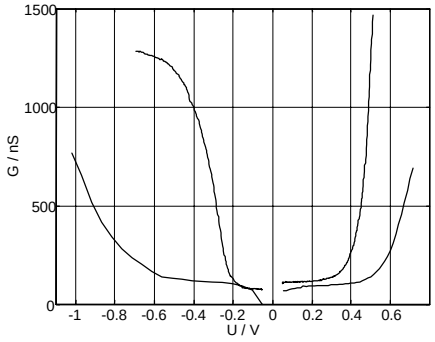

Figure 7: Current/voltage relationship for voltage clamp with different slew rate of the stimulus. From left: (-100 -5 2.5 28) V/s.

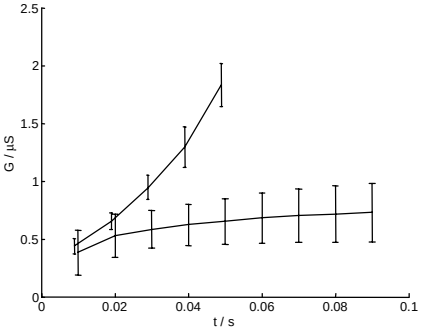

Figure 8: Membrane conductance during long lasting voltage across the plasma membrane. Upper curve U = 500 V, N = 7, (depolarization), lower curve U = - 500 mV, N = 22 (hyper-polarization). The error bars show the standard deviation.

However, if a moderate voltage is clamped across the membrane (500 mV in Fig.8), an electrically stable membrane appears during hyperpolarization, while the conductivity in a depolarized membrane increases, resulting in complete destruction of the cell.

Discrete jumps in conductivity With sufficiently high trans-membrane voltages (> 0.8V) dramatic structural changes on the membrane can occur instantaneously (μs-range). As shown in Fig.9, such a step can reach several hundred nS. If this is interpreted as the appearance of a huge single pore, it exceeds a diameter of 10 nm. These discrete steps are very frequent at hyperpolarization and during voltage-clamp. They appear, however, also under current-clamp and even during depolarization. Moreover, vesicle formation (blebbing) at the cell membrane occurs very frequently during hyperpolarization but only for a destructive condition during depolarization.

Discussion

The CVC depends on the polarity of the stimulus and also on the temporal development of the stimulus. Since we investigated the membranes of living cells,

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voltage gated channels and active responses of the cell may influence our results. Given the voltage range below 100 mV, where active responses of the cell matter, one would already expect dramatic changes in the membrane behavior in this range. According to Fig. 3 and Fig. 4, even a stimulus of 200 mV does not dramatically change the electrical behavior of the membrane. From the current-voltage behavior below the critical level for electroporation, the passive electrical properties of the membrane (conductance, capacitance) have been estimated. The area-related conductivity of the cell membrane increases by a factor of 2 to 5 (30 – 50 mS/cm^2) compared to the pre-stimulus values (5 - 20 mS/cm^2). In addition, the membrane capacitance reaches 6 μF/cm^2, which is six times more than the capacitance in resting condition. The conductance and capacitance increase is mostly reversible and assumed to be due to the modulation of the Guy-Chapman – layer in the electric field.

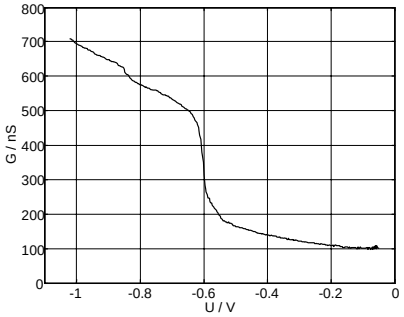

Figure 9: Step increase of the conductivity during hyper-polarization

Once electroporation occurs, the conductivity of the membrane increases dramatically. Although it has no effect under voltage clamp conditions, the electric field within the membrane decays during current clamp. This means that current clamping exhibits a negative feedback between the stimulus and the driving force for structural changes and trans-membrane transport. This has practical relevance for electroporation of cells in low conductive media. As soon as the membrane is electroporated, the voltage divider between the membrane and the surrounding electrolytes causes a decay of the electric field. Although there is no direct positive feedback during voltage clamp, the longer the electric field lasts above the critical level, the greater the probability of further pore creation [3]. Moreover, the increased current at the same transmembrane voltage causes Joule heating which, in turn, reduces the critical voltage for electroporation, forcing further pore creation. This may be important for cell suspensions in highly conductive media. However, since the medium shunts the cell, electroporation will not dominate the electrical behavior of the suspension. This does not apply if the medium is heated only by long-lasting strong pulses. If the cells are densely packed with a highly conductive extra-cellular medium, as is found in muscles or especially within the myocardium, Joule heating due to electroporation can be important. Even with electroporation, the membrane conductivity is small compared to that of the extra-cellular medium, yielding only negligible decay in transmembrane voltage. This has even greater practical relevance since therapeutic application of electroporation involves low field (200 V/cm), long lasting pulses (10 – 200 ms) in highly conductive tissue. This does not hold for a very high electric field (> 10 kV/cm), since, under these circumstances the cell membrane shows a conductivity greater than one would expect, by replacing all lipids with electrolytes.

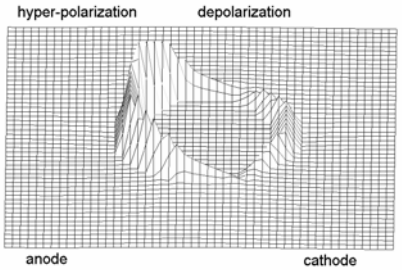

Figure 10: Simulation of the field strength for a cell in a homogenous field. Hyperpolarization occurs at the anode side while depolarization is found at the cathode side.

It is observed in numerous studies, that most of the effects of the electric field occur at the anode side of the cell, which is the side undergoing hyper-polarization. However, the conductance increase due to electroporation is much more pronounced at the opposite side. This, however, is measured in whole cell clamp arrangement. Using the experimental CVC, it is possible to obtain approximate values of the transmembrane voltage using a finite element simulation. Fig. 10 shows features of the simulation model.

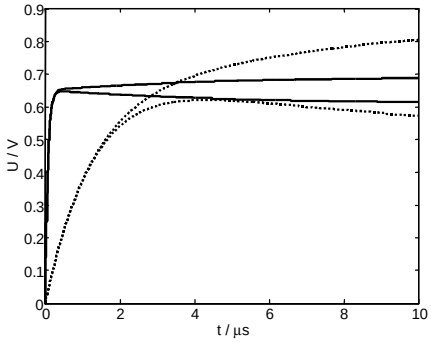

Figure 11: Estimation of the trans-membrane voltage in an electric field of 750 V/cm for a cell with 8 μm radius. Solid lines: high medium conductivity (1 S/cm), dashed line low medium conductivity (10 mS/cm). The conductivity of the cytosol was set to 1.4 S/cm and the membrane capacitance was constant at 1 μF/cm^2. The experimental data shown in Fig. 6 were used to calculate the voltage dependent membrane conductivity. No time varying events were simulated.

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A circular-shaped cell is placed in a homogeneous electric field. At t=0 s, an electric field is turned on and the increase of the transmembrane voltage is calculated in time steps of 10^{-9} s, up to 10 μs.
Two runs were done, one with a high and one with a low conductive medium surrounding the cell. All parameters were held constant with the exception of the membrane conductance. For the highly conductive medium, the data for the voltage clamp were used, while the current clamp data were used for the run with the low conductive medium (Fig.11).

These results show several important features: (1) the voltage at the hyperpolarization side is higher than at the opposite one, (2) the difference is less for a highly conductive medium, and (3) the charging during the first nanoseconds depends on the capacitance, rather than the conductance, of the membrane. This, however, is beyond the scope of this study. The longer charging time for the low conductivity medium is not discussed here, since this is common knowledge.

Our hypothesis is that the permeability for small ions is not important for structural changes at the membrane. The only important parameter is the electric field within the membrane and the time where a critical field strength was exceeded. As estimated from the single cell experiments, but also from the simulation, the field strength at the hyperpolarization side is higher than that at the depolarization side. This increases the probability for structural changes. Due to the voltage divider between membranes and the surrounding medium, for achieving an electroporation condition, a lower applied field is necessary for high conductive media. This is consistent with the observation that tissues and pellets are easier to electroporate, even if the factor of 1.5 for the membrane field at the pole caps of single cells in suspension is taken into account.

As shown in fig. 8, a long lasting stimulus below the critical field strength for immediate structural changes causes an increase in membrane permeability as well. However, this occurs more at the depolarization side. If an entire cell is considered, the conductance increase at one side will increase the transmembrane voltage at the opposite one, due to the voltage divider. Consequently, the depolarization side protects itself by lowering the membrane field. The potentially higher current is not important, since both the hyperpolarization and the depolarization side are electrically in series. But why should the hyper-polarization side show a larger permeability? As shown in fig. 9, frequent discrete steps in conductance increase occur at the hyperpolarization side. These steps are probably due to the creation of large pores, limited by the spectrin network of the cell cortex. This seems contradictory to the curved membrane of a cell; however, given the mechanical behavior of the cell cortex, the membrane between the integrenes does not show the forces in a curved membrane but behaves rather like a planar membrane. Another hypothesis is that the membrane within the electric field becomes unstable and undergoes micelle formation, since the repulsive interaction between water and the tail groups is lowered by the electric field. This becomes even more likely when the membrane is charged at a very high slew rate, so that even the self-protecting lowering of the membrane conductance does not prevent a very high field within the membrane. Under these circumstances, an even greater effect exists, forcing water into the membrane between the head-groups and thereby making micelles a favored structure of the lipids. As evident from impedance measurements, a recovery of the membrane takes place, suggesting that even large leaks do not necessarily cause the death of the cell.

Conclusion

It is not the dramatic conductance increase that forces large scale structural changes on the membrane level, but rather, the elevated electric field for a critical time. The depolarizing side in cell electroporation protects itself from dramatic structural changes by increasing its permeability to small ions, thereby weakening the electric field. This effect is absent at the hyperpolarization side, causing dramatic changes after a high electric field persists for a critical time. These changes may involve a mechanical rupture of the membrane or micelle formation, solubilizing the membrane. Since the electric field, not the current flow, is responsible for structural changes, one should use high electrical stimuli with a high slew rate, but short pulse duration, for cell manipulation.

Acknowledgments

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