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Ion transport into cells exposed to monopolar and bipolar nanosecond pulses

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Abstract

Experiments with CHO cells exposed to 60 and 300 ns pulsed electric fields with amplitudes in the range from several kV/cm to tens of kV/cm, showed a decrease of the uptake of calcium ions by more than an order of magnitude when, immediately after a first pulse, a second one of opposite polarity was applied. This effect is assumed to be due to the reversal of the electrophoretic transport of ions through the electroporated membrane during the second phase of the bipolar pulse. This assumption, however, is only valid if electrophoresis is the dominant transport mechanism, rather than diffusion. Comparison of calculated calcium ion currents with experimental results showed that for nanosecond pulses, electrophoresis is at least as important as diffusion. By delaying the second pulse with respect to the first one, the effect of reverse electrophoresis is reduced. Consequently, separating nanosecond pulses of opposite polarity by up to approximately hundred microseconds allows us to vary the uptake of ions from very small values to that obtained with two pulses of the same polarity. The measured calcium ion uptake obtained with bipolar pulses also allowed us to determine the membrane pore recovery time. The calculated recovery time constants are on the order of ten microseconds.

Keywords

nanosecond pulses; monopolar and bipolar pulses; electroporation; calcium ions; electrophoresis; diffusion; pore recovery

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1. Introduction

The effect of the shape of electrical pulses on the permeabilization of biological cell membranes has been the topic of multiple experimental studies [1–7]. It must be noted that almost all of the studies were performed with pulses of millisecond duration. Only, one of the cited manuscripts [1] reports the use of microsecond pulses – bipolar pulse trains of 8.33 μ s pulse duration for a total pulse train duration of 400 μ s. The conclusion of these studies was that bipolar pulses have a positive effect on electroporation compared to monopolar pulses. The reason given was generally that bipolar pulses compensate for the asymmetry of electroporation with monopolar pulses.

Recently, however, studies with much shorter pulses, 300 ns and 600 ns, applied to Chinese Hamster Ovary (CHO) cells showed surprisingly that nanosecond, bipolar pulses are less effective at electroporation of the plasma membrane, contrary to the results obtained with longer pulses [8]. They also were found to be less effective in increasing intracellular calcium concentration. Studies with even shorter pulses, 60 ns and 300 ns, with CHO cells confirmed these results: an attenuation of the uptake of calcium, after pulsed electric field (nsPEF) application - when a pulse of opposite polarity was applied immediately after the first pulse [9]. In addition, measurements with bipolar pulses, separated in time, showed that the attenuation became less and less pronounced when the second pulse with opposite polarity to the first one was delayed by times on the order of microseconds.

The results of these studies performed with 300 ns pulses are shown in the histogram in Fig. 1 for two electric field intensities: 5.6 kV/cm and 7.5 kV/cm [9]. In order to exclude the pulse-induced calcium release from the endoplasmic reticulum, the cells were treated with CPA (Cyclopiazonic Acid). With just a single monopolar pulse of 300 ns applied, the total calcium uptake (maximum value) of a CHO cell was measured as 2.1 μ M at 5.6 kV/cm. It increased to 3.8 μ M at 7.5 kV/cm. When bipolar pulses, two identical 300 ns pulses with opposite polarity, were applied to the CHO cells, the calcium uptake was found to be less, even though the total electrical energy had doubled. For the case where the second pulse followed the first one immediately the calcium uptake was lower by more than one order of magnitude (white bars). With increasing time between the two bipolar pulses, 0.4, 1, 5, and 10 μ s, this ratio approached that of single monopolar pulses but did still not reach the value expected for two independent monopolar pulses – twice that of the single monopolar pulse.

For CHO cells stimulated by nanosecond pulses the endoplasmic reticulum (ER) is the sole significant source of intracellular calcium [10]. It is therefore obvious that the measured cytosolic calcium, for the case that the ER is depleted by using CPA, can only be delivered from the medium through the plasma membrane. The ions enter the cell interior through nanopores which are generated in the membrane when intense nanosecond pulses are applied [11,12]. Transport of molecules through the plasma membrane for nanosecond pulses (nsPEF), has been discussed in detail in reference [13]. However, the authors had to rely on data on pore recovery which were obtained from pulse experiments with longer pulses, and thus concluded that the dominant transport mechanism was diffusion after the pulse, rather than electrophoresis. Our experimental data provide a more consistent base for assessing the role of the two transport processes, electrophoresis and diffusion. We are using

only a subset of the results reported in reference [9] in our study, focusing solely on the result where CPA was used to deplete calcium from the ER, which otherwise would be released from it. The results, obtained with 300 ns long monopolar and bipolar pulses (Fig. 1, insert), therefore represent the sum of the calcium ions which passed through the plasma membrane and have accumulated in the cell after pulsing. The experimental results, together with information on nanoporation and the mobility of molecules passing through the membrane, allow us, not only to obtain a more accurate evaluation of the role of the charge transport through the membrane for monopolar and, at least qualitatively, for bipolar pulses, but also provide information on pore recovery times after nanosecond pulse exposure.

2. Theoretical Considerations

2.1 Monopolar Pulses: Electrophoresis versus Diffusion

The physical processes which govern transmembrane transport through electroporated membranes are diffusion and electrophoresis, with electrophoresis also known as electrodiffusion or drift. Active mechanisms of calcium regulation, such as active calcium pumping out of the cell, are considered to be of minor importance and will be neglected in the following. Diffusion describes the motion of any particle charged or not in the direction from higher concentration to lower concentration. It is a process which occurs during and after pulsing the cell as long as the membrane is porated. In our case, where the extracellular concentration of calcium (2 mM) is always large compared to the cytosolic concentration, even after the influx of calcium, this flux is in one direction only: from the medium into the cell. The second transport mechanism is electrophoresis. Electrophoresis describes the motion of charged particles, ions, under the influence of an electric field, E . For positively charged ions, such as calcium, we have, after electroporation is established and as long as the pulse is applied, a electrophoresis flow into the cell at the anodic side of the cell, and out of the cell at the cathodic side.

If we just focus on the calcium flow into and out of the cell, rather than the total current density due to all ions, we can write the following balance equation which states that the measured amount of calcium, its total charge, Q_{Ca} , respectively, in the cell is due to the transport of calcium ions from the time the pulse is applied and electroporation is established to the time the cytosolic calcium is measured:

$$Q_{Ca} = \int_0^\tau J_{ECa}(t) A(t) dt + \int_0^\tau J_{DCa}(t) A(t) dt + \int_\tau^T J_{DCa}(t) A(t) dt \quad [1]$$

The first integral in Eq. 1 describes the amount of calcium charge which is carried by electrophoresis into the cell, with J_{ECa} being the current density. A is the area of the cell membrane through which the calcium ions flow. Since electrophoresis is only present when the pulse(s) is (are) applied, the upper limit in the integrals is the total duration of the pulse(s), τ . The second integral describes the amount of calcium carried by diffusion into the cell during the time of the pulse(s) and the third integral describes the amount of calcium carried by diffusion into the cell after the pulse, up to the time, T , when the calcium amount inside the cell is measured. However, to use the time of measurement as this upper limit in time is only valid if the pores are open for times equal or longer than the time of measurement. If

they close earlier than the upper limit in time, it needs to be replaced by the pore closing time.

Since the extracellular calcium concentration with 2 mM is always larger than the cytosolic calcium concentration, diffusion in our case is always directed into the cell, whereas electrophoresis will drive the ions in the direction of the electric field vector either into or out of the cell, depending on the direction of the electric field with respect to the membrane surface. It was shown (Appendix A) that the ratio of the integrands 2 and 1 is very small, just 1.3%, independent of pulse duration and pulse amplitude. That means diffusion during the pulse can be neglected compared to electrophoresis, and equ. 1 can be reduced to:

$$Q_{Ca} = \int_0^{\tau} J_{ECa}(t) A(t) dt + \int_{\tau}^{\tau_c} J_{DCa}(t) A(t) dt \quad [2]$$

In addition we have in Eq. 2 replaced the time of calcium measurement, T in the upper limit of the diffusion integral with the pore closure time, τ_c , assuming that it is less than T.

Calculating the total calcium charge transferred into the cell through electrophoresis from the anodic side (there is no transfer out of the cell at the cathodic side, since the mobile calcium concentration in the cytosol is zero) is obtained by solving the first integral in Eq. 2. The first variable in the integral is the calcium current density. It is shown in Appendix B that for the medium used in our experiments for 300 ns pulses and an E of 5.6 kV/cm the calcium current density is 0.77 A/cm², and for an electric field intensity of 7.5 kV/cm the value is 1.03 A/cm².

We assume that this value is independent of time, which is a given for a square wave pulse, but also independent of the location at the electroporated cell surface, a reasonable assumption for the case that extracellular and intracellular resistivities are equal. In this case we can pull the current density for the time of pulse application in front of the integral:

$$Q_{ECa} = J_{ECa} \int_0^{\tau} A(t) dt \quad [3]$$

The second variable in this transport integral is the electroporated area through which the calcium current can flow. This area increases with time and, at constant applied electric field, E, approaches a maximum which is dependent on the value of E. The calculation of the temporal development of the electroporated area is described in Appendix B.

Let us compare these computed results with measured results. With an electric field, E, of 5.6 kV/cm, the charge carried by calcium ions into the cell by the end of the 300 ns pulse is $Q_{ECa} = 5.48 \cdot 10^{-13}$ As (Appendix B). The number of calcium ions entering the cell is therefore $N = 1.7 \cdot 10^6$. With a cell radius of $a = 9 \mu\text{m}$ (assuming a spherical cell shape), and consequently a cell volume of $V = 3.1 \cdot 10^{-9} \text{ cm}^3$, the calcium concentration in the cell is $n = 5.5 \cdot 10^{14} \text{ cm}^{-3} = 0.86 \mu\text{M}$. For E = 7.5 kV/cm and the same pulse duration, the calcium concentration in the cell at the end of the pulse is $n = 1.10 \mu\text{M}$. Comparing these values with the measured ones (Fig. 1) we see that this constitutes less than one fourth of the measured calcium concentration. That means that the second integral in Eq. 2, which describes the diffusion after the pulse, has a value of not more than four times the value as the electrophoresis integral. It is definitely not orders of magnitude higher.

This importance of electrophoresis for the uptake of calcium ions seems to be even more pronounced for shorter pulses. Results of calcium measurements for 60 ns pulses are shown in Fig. 2. Using the same type of calculations as for the 300 ns pulses, at an electric field strength of 28 kV/cm a value which was chosen, based on a scaling law for bioelectric effects of nanosecond pulses [14], the calculated calcium concentration due to electrophoresis is $n_{Ca} = 0.83 \mu\text{M}$, exceeding the measured value of the total calcium concentration of $0.25 \mu\text{M}$ by a factor of about three. Considering, however, that we have assumed a constant electroporation voltage of 1 V, whereas measurements of the anode side voltage with 60 ns pulses showed voltages of up to 1.6 V for pulses of this duration [15], and its influence on pore opening time and total poration area, the calculated results will be less than stated above. The conclusion from the results of this calculation is that electrophoresis is at least as important as diffusion in the uptake of calcium, and likely other light ions, into cells. This is in stark contrast to earlier transport modeling results [13], which concluded (based on an assumed long pore recovery time) that the effect of diffusion exceeds that of electrophoresis by orders of magnitude.

2.2 Bipolar Pulses: Forward and Reverse Electrophoresis

Dependent on the direction of the electric field with respect to the cell surface, it can generate a flow into or out of the cell. In the case of a monopolar pulse and a zero concentration of calcium ions in the cell, there is only a flow into the cell. For positive ions, such as calcium ions, this inflow is through the anode directed membrane. With cathode and anode changing positions for the case of bipolar pulses, these directions are reversed when the polarity of the pulse changes.

These processes are schematically illustrated in Fig. 3. When the first pulse is applied, calcium ions will drift into the cell on the anode side (left) indicated by the arrows. Since there are no mobile calcium ions in the cell after treatment with CPA, the effect on the cathode side is just a shift in calcium concentration away from the cell. That means after the first pulse there will be a layer with increased calcium concentration at the anodic side inside the cell, with a thickness given by the electrophoresis length, L_E , the average distance over which a calcium ion will move under the influence of an electric field, E . This electrophoresis or drift length is given as:

$$L_E = D_{Ca} 2eE\tau / kT \quad [4]$$

with D_{Ca} being the calcium diffusion coefficient, e , the elementary charge ($2e$ is the total charge for calcium ions), τ , the pulse duration (of square wave pulse) and kT , the thermal energy (k is the Boltzmann constant and T the absolute temperature). With D_{Ca} given as $0.79 \cdot 10^{-5} \text{ cm}^2/\text{s}$ [16], and with kT/e being 26 mV at room temperature, the electrophoresis length for a 300 ns pulse at an electric field of 7.5 kV/cm is 9.1 nm. At the cathode side, but outside the cell, a calcium depleted boundary layer of the same thickness, L_E , will be created. When the electric field is reversed, the flow is also reversed: The calcium ions at the former anode side are driven out of the cell, and those in the vicinity of the cell at the former cathode side fill the depleted space at this side. The net effect through electrophoresis would, under ideal conditions (second pulse of opposite polarity follows the first one

immediately and diffusion during the pulses can be neglected), be zero. After the pulse the only transport process is diffusion radially into the cell.

What happens when the pulses are separated in time? The amount of calcium swept out of the cell at the anode side, when the pulse of opposite polarity is applied, will decrease with increasing separation between the positive and negative polarity pulses. The reason for a weakening of this sweep-out effect is the diffusion of the calcium ions away from the membrane into the interior of the cell, as shown in figure 4a (left), leaving a volume with lower concentration behind. This assumes that diffusion through the membrane does not replenish the calcium in this layer. We will show later (Appendix A) that this is a reasonable assumption. Since the electrophoresis length does not change when the pulses are separated, only a fraction of the calcium concentration will be swept out. This fraction will become less and less with increasing pulse separation until eventually the results of the calcium measurements resemble that of two independent pulses. A schematic sketch of the time dependent calcium distribution at the anode side after the first pulse (Fig. 4b, left) is used to illustrate the diminishing effect of sweep-out when the bipolar pulses are separated.

At the cathode side (Fig. 4a,b, right) we have a different situation. When the first pulse is applied, the calcium ions at this side are drifting away from the membrane. This leaves a calcium depleted boundary layer with a width of the electrophoresis length, L_E , close to the membrane. When the voltage is reversed, the calcium ions electrophoresis back into the depleted zone and fill it up again. The diffusion front fed by a high concentration source of extracellular calcium advances with time, t , according to:

$$L_D = (D_{Ca} t)^{1/2} \quad [5]$$

For a separation time of 1 μ s that is a distance of 2.8 μ m for Ca^{2+} , which is large compared to the electrophoresis or drift length. The second pulse with opposite polarity will then sweep those calcium ions which have diffused into the boundary layer through the porated membrane into the cell. For short bipolar pulses, which follow each other immediately, diffusion doesn't play much of a role. In this case the reverse pulse effect at the initially cathodic side does not lead to a considerable transfer of calcium ions into the cell at this side. However, for long pulses and/or with increasing separation of even short pulses, the volume on the initially cathodic side fills up through diffusion before the second pulse is applied. In these cases an increasing fraction of calcium ions is swept into cell, diminishing the sweep-out effect at the opposite side.

Of course, the reverse electrophoresis effect which causes a reduction in cytosolic calcium concentration would be overshadowed by diffusion, which is a unidirectional process, if the pores were open for a long time compared to the pulse duration. Actually, most of the experimental data list pore closure times which vary between milliseconds and hours [17–21]. An overview of experimental results for pore recovery times obtained before 1991 is given in reference [17]. Some newer results are listed in references [18–21]. Only one publication [22] provides results which are directly related to membrane recovery after nanoporation. In this study where the conductivity of black lipid bilayers was measured after

submicrosecond pulses were applied, a decrease of the membrane conductivity on a time scale of microseconds was measured. However, based on the majority of available data on recovery time, an average value of one second was used in a previous modeling study dealing with membrane transport during and after nanosecond pulse application [13]. Consequently it was assumed that diffusion, rather than electrophoresis is for nanosecond pulses the dominant ion transport mechanism across the cell membrane. This is in line with studies where 100 microsecond long pulses were used, and where also the effect of electrically driven transport was considered to be small enough to be neglected in a model which describes small molecule uptake after electroporation [23].

In our case we don't have to rely on data on pore closure times obtained under different pulse conditions on different cell types. The experimental results, shown in Fig. 1, together with information on the physical parameters of the cell and particularly the cell membrane allows us to calculate the effect of electrophoresis and diffusion on the measured calcium concentration. By using these data it could be shown that electrophoresis is at least as important as diffusion in the case of nanosecond pulses, a result which supports our hypothesis, that reverse electrophoresis is the cause for the reduced ion uptake in case of bipolar pulses. As a consequence of this result it will also be shown that the relevant characteristic pore closure time in our case is much less than milliseconds and therefore comparable to that measured by Benz and Zimmermann [22].

2.3 Pore Recovery Time

From the results of the calculations on the nanosecond pulse facilitated uptake of calcium in CHO cells in section 2.1, we conclude that diffusion after the pulse has an effect on the uptake which is for 300 ns pulses on the same order or for 60 ns even less than electrophoresis. Since the first integral in Eq. 2, which describes electrophoresis, has been solved (Appendix B), and since we know through measurements the total charge of calcium ions in the cell, Q_{Ca} , we can solve Eq. 2 for the second term, which describes diffusion after the pulse. Assuming that the diffusion current is constant during the time of pore opening, we obtain the pore recovery time, τ_{pr} :

$$J_{DCa} A (\tau_{pr} - \tau) = Q_{Ca} - Q_{ECa} \quad [6]$$

The diffusion current density, J_{DCa} , can be expressed in terms of the known electrophoresis current density, J_{ECa} , (Eq. A7), taking the porated cell surface area into account. The surface area of cell is $A_c = 4\pi a^2$, which for a radius of $a = 9 \mu m$ results in a value of $1.02 \cdot 10^{-5} cm^2$. However, since θ , the angle which defines the opening of the membrane is, according to Eq. A14 less than 90° (81° for 300 ns and 5.6 kV/cm, and 84° for 300 ns and 7.5 kV/cm), the porated surface area is slightly less: $0.94 \cdot 10^{-5} cm^2$ for 300 ns pulses at 5.6 kV/cm and $0.7 \cdot 10^{-5} cm^2$ for 300 ns pulses at 7.5 kV/cm. Using these data, Eq. 6 can be solved for the difference in pore recovery time and pulse duration, τ :

$$(\tau_{pr} - \tau) = 2Ed (Q_{Ca} - Q_{ECa}) / J_{ECa} A (K_{DCa} / K_{ECa}) (kT/e) \quad [7]$$

A is the cross-sectional area, and K_{DCa} and K_{ECa} are the partition factors for calcium diffusion through the membrane during and after the pulse [13]. For the monopolar, 300 ns long pulses with amplitudes of 5.6 kV/cm and 7.5 kV/cm, the calculated pore closing time is 140 and 210 μ s, respectively. For the 60 ns pulses, the pore recovery time cannot be obtained by this method, since the calculated electrophoresis charge is larger than the measured total charge.

The results of these calculations are obviously not very accurate, since they rely on the difference between a measured and a calculated value (electrophoresis), rather than on a measured result. A better way to obtain information on the pore recovery time is to utilize the measured values of the calcium charge for the bipolar pulses which follow each other without temporal separation. Under ideal conditions - each ion which drifts into the cell during the first pulse is driven out by the opposite polarity pulse - the electrophoresis part of the charge would be zero. The measured, nonzero, value can therefore only be attributed to the diffusion during and after the pulse application. Even in the more realistic situation, where not all of the calcium ions are swept out by the second pulse, this measured value would still provide us with information on the upper limit in diffusion generated charge inside the cell. So the resulting closure time can only be shorter than the calculated one using this concept, not longer. According to these deliberations, the measured calcium charge generated by a bipolar pulse with a total duration of 2τ , Q_{bpCa} , represents therefore the upper limit of calcium charge carried into the cell by diffusion, during and after the pulse, Q_D .

$$Q_{bpCa} < Q_D = A_c (J_{D1} 2\tau + J_{D2} \tau_{pr}) \quad [8]$$

where τ_{pr} is the pore recovery time. The diffusion current densities during and after the pulse application, J_{D1} and J_{D2} , respectively can as before be expressed in terms of the electrophoresis current density, J_{ECa} (Appendix A) taking the area of the porated cell surface into account. Using Eq. A7 we obtain:

$$Q_{bpCa} < 4\pi a^2 \{ J_{ECa} (kT/e) / (2Ed) 2\tau + J_{ECa} (K_{DCa}/K_{ECa}) (kT/e) / (2Ed) \tau_{pr} \} \quad [9]$$

For the 300 ns bipolar pulses at 5.6 kV/cm, the experimental value of Q_{bpCa} is 0.15 μ M, that of 7.5 kV/cm is 0.5 μ M (Fig. 1). The value of the pore recovery time, τ_{pr} , for the 5.6 kV/cm case is then less than 6 μ s, that for 7.5 kV/cm, less than 27 μ s. These values are well below the range of reported pore closure times which range from milliseconds to hours. They agree, however, within an order of magnitude with the measured decay time constants for the conductivity of membranes subjected to nanosecond pulses which was measured as 3 μ s [22].

3. Discussion

The validity of our hypothesis, that the reverse electrophoresis motion of ions, when, after a first monopolar pulse a second pulse with opposite polarity is applied to cells, is depending heavily on the assumption that electrophoresis rather than diffusion is the dominant transport effect for nanosecond pulses. By using the quantitative results of calcium measurements in

CHO cells, and information on the physical parameters of these cells and the suspension, for 300 ns pulses, it could be shown that electrophoresis is at least as likely as diffusion to contribute to the transport of calcium into the cells. Even the uncertainty in data, particularly in the values of the diffusion coefficients in the cytoplasm [24] and possibly in the partition factors [13], is likely not affecting this conclusion. That electrophoresis is the dominant effect in ion transport through electroporated membranes not only for nanosecond pulses, but longer pulses as well, is indicated by the results of a theoretical study [25]. Numerical calculations of molecular uptake of calcium ions by CHO cells, identical in cell type and ion species to those used for studies described in reference [9], and in this manuscript, however at much longer pulse durations of 6 ms, showed also that diffusion plays a secondary role to electrophoresis in ion transport across the electroporated membrane.

A consequence of the dominance of electrophoresis over diffusion, at least for nanosecond pulses, is a rather short pore closure time for nanopores. According to our calculation it is on the order of 10 μ s, much shorter than the often assumed pore closure time on the order of a second, which is based on experimental results obtained with a temporal resolution less than our calculated pore closure time. The only measurement which was performed with temporal resolution in the nanosecond range after submicrosecond pulses were applied to black bilayer membranes [22] shows similar results as our modeling results: a decrease in membrane conductivity with a time constant of microseconds. This result does not invalidate other experimental results which indicate much longer pore closure times, particularly the measurements described in references [21,26], where, through patch-clamp studies, the resistance of the plasma membrane after exposure to 300 ns pulses was found to increase with a time constant of tens of minutes. It only shows that a single time constant cannot be used to describe the entire temporal development of membrane recovery [18,27].

The results of these measurements [21,26], that of Benz and Zimmerman [22], and our modeling results which are based on calcium uptake, are plotted in a double logarithmic diagram (Fig. 5). By graphing it this way, we were able to cover the immense range of conductivity and recovery time. It shows that the pore recovery, expressed as the decrease of membrane conductivity, can be described by a combination of short and long time scale effects. The uptake of ions is given by the integral over these curves. It is obvious from this graph, that the fast uptake of ions, although at an extremely high rate, might not surpass the uptake of ion for minute-long pore recovery times, even if it occurs at a much lower rate. In other words, a pore which stays open for 100 seconds may allow the same transport of ions as 10 million pores which just stay open for 10 microseconds.

With increasing temporal distance between bipolar pulses, the effect of diffusion further into the cell at the anode side and the partial elimination of the boundary layer at the cathode side lead to a reduction in the intensity of the electrophoresis effect. With separation times between the two pulses of opposite polarity exceeding the pore closure time, the uptake of molecules will approach that measured with two independent monopolar pulses. This fact allows us to utilize the shift in time between bipolar pulses to control molecular uptake from a minimum value determined by the relatively minor diffusion processes during pulsing, to twice the uptake of single monopolar pulses. This holds not only for calcium but for other

ions as well. It also holds for calcium release from subcellular structures, such as the ER. This has been shown in experiments, where no CPA was used [9].

The electric field of electromagnetic waves can be considered as a periodic sequence of bipolar pulses, and therefore similar effects as described above could be expected. The condition is that the electric field amplitudes of these pulses are high enough to generate pores in cells. An exciting consequence of this hypothesis is the use of a set of single frequency electromagnetic waves. By choosing their frequencies and amplitudes through Fourier synthesis, we can create temporal structures with positive and negative peaks, separated by more than one period. This allows us to generate periodic electric fields which resemble the structure of a sequence of bipolar pulses similar to those shown in Fig. 1 with pulses separated by a certain time. By modulating the frequencies of these electromagnetic waves, and consequently, the temporal structure of the electric fields, we might be able to control the uptake of molecules into cells. The use of nanosecond, bipolar pulses might therefore not just allow us to learn more about transport processes through the cell membrane, and pore closure times, but also may lead to new ways of controlling the uptake of ions, and consequently affect cell functions, including cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A: Electrophoresis and Diffusion

For charged particles, ions, diffusion results in a current density, which can be expressed as the sum of all diffusion current densities, J_D , of the various ions (characterized by the letter i) in and outside the cell:

$$J_D = - \sum H_{iD} K_{iD} [q_i D_i (dn_i/dx)] \quad [A1]$$

where q_i is the charge of the ions of species i , D_i is their diffusion coefficient, n_i is density of the ion species, and (dn_i/dx) the gradient in the membrane. Assuming a linear change in concentration across the membrane, the gradient can be expressed as the difference in concentration in and outside the cell, and dx can be replaced by the membrane thickness, d .

H_{iD} is the so-called hindrance factor for diffusion through the membrane. It serves as a measure of the impediment of the ion(s) flow through the pore based on the size of the ion (and neutral particles) relative to the diameter of the electropore. Obviously, if the pore size is large compared to the diameter of the ion, this factor approaches the value of 1. If the diameter of the ion approaches that of the pore, the current flow will be reduced, and H will approach the value zero. Since a nanoelectropore is assumed to have a diameter of approximately 1.6 nm [13], for the light ions, including calcium, H_{id} can be set to 1.

K is the so-called partition factor. It describes the effect of coulomb interaction of the ions passing through the membrane with the charge distribution on the pore surface. It is therefore dependent on the charge of the ion, the pore radius and the membrane voltage. The higher the charge of the ion, and the higher the membrane voltage, the lower is the partition factor. Values for the case that the membrane voltage has a value of 1 V and the pore radius is 0.8 nm, are 0.91 for singly charged ions, and 0.83 for doubly charged ions. After the pulses, with the plasma membrane voltage approaching a value small compared to 1 V, the partition factor for singly charged molecules is 0.51, that for doubly charged ion, it is 0.053 [13].

If we only consider the diffusion current of calcium ions, Eq. A1 changes to:

$$J_{DCa} = -H_{CaD} K_{CaD} 2e D_{Ca} (dn_{Ca}/dx) \quad [A2]$$

with e being the elementary charge. Since calcium is doubly charged we take q as 2e. We can simplify the equation further: since the concentration of extracellular calcium with 2 mM exceeds that of the intracellular contraction for any time, we can replace the gradient by n_{Ca}/d , leading to:

$$J_{DCa} = -H_{CaD} K_{CaD} 2e D_{Ca} n_{Ca}/d \quad [A3]$$

The second transport mechanism is electrophoresis. Electrophoresis describes the motion of charged particles, ions, under the influence of an electric field, E. The total electrophoretic current density, J_E , which is generated by all the various ion species, characterized by the subscript i, is given as:

$$J_E = \sum H_{iE} K_{iE} [q_i^2 D_i n_i E / kT] \quad [A4]$$

where kT is the thermal energy; for room temperature it is 26 meV, with e being the elementary charge of an electron. For the high electric field intensities with which we deal, we can neglect magnetic effects for nanosecond pulses, and only consider the effects of the externally applied electric field. If we just consider calcium electrophoresis the current density can be expressed as:

$$J_{ECa} = H_{CaE} K_{CaE} 4e^2 D_{Ca} n_{Ca} E / kT \quad [A5]$$

Using Eq. A3 and Eq. A5, the ratio of the electrophoresis current density and diffusion current density (H and K are the same for diffusion and electrophoresis during the pulse) is:

$$J_{DCa}/J_{ECa} = (kT/e) / (2Ed) \quad [A6]$$

With kT/e being 0.26 mV and Ed = 1V, the ratio is 1.3%. That means diffusion during the pulse can be neglected compared to electrophoresis.

Comparing the integrands of the two remaining integrals gives us the following result:

$$J_{D_{Ca}}/J_{E_{Ca}} = (K_{D_{Ca}}/K_{E_{Ca}}) (kT/e) / (2Ed) \quad [A7]$$

In this case we have to take the differences in the partition factor into account, since during pulsing (electrophoresis) K is larger than after pulsing (diffusion). With $K_{D_{Ca}} = 0.053$ and $K_{E_{Ca}} = 0.83$, the ratio of the two integrands is 0.083%, even smaller than during the pulse. However, since the time after the pulse, before the pores are closing, and where electrophoresis is zero, but diffusion continues, is likely much longer than the pulse duration time, diffusion can possibly contribute much more to the final calcium concentration in the cell. E.g., if the pores don't close before the time of the measurement (which is on the order seconds), for nanosecond pulses, diffusion would far exceed the effect of electrophoresis. This has been pointed out in reference [13], however, it is only valid if the pore closing time exceeds the pulse duration by many orders of magnitude.

Appendix B: Solution of the Electrophoresis Integral for a Monopolar Pulse

The first integral in Eq. 2 describes the contribution of electrophoresis to the uptake of calcium. In order to calculate the calcium uptake through electrophoresis, we need to express the integrand $J_{E_{Ca}}(t) A(t)$ in terms of measurable quantities. We can determine the drift current density in the medium by using Ohms law:

$$J = E/\rho \quad [A8]$$

where ρ is the resistivity of the medium. This value has been measured as 80 Ωcm . The resistivity of the cytosol has not been measured. It is often assumed to be 100 Ωcm . Lower values have been reported by Foster et al [28]. We assume, for simplicity, that the resistivity of the cytosol lies between these values at 80 Ωcm . The current density outside and inside the electroporated cell is then identical, and so must consequently also be the drift (electrophoretic) current density in the membrane.

The current density of the calcium flow into the cell, J_{Ca} , assuming that the diffusion current during the pulse can be neglected, can be expressed in terms of the measured current density, J , multiplied with the computed ratio of calcium current density to drift current density (Eq. A5 and Eq. A4):

$$J_{Ca} \approx J_{E_{Ca}} = (E/\rho) (J_{E_{Ca}}/J_E) = (E/\rho) H_{CaE} K_{CaE} 4 e^2 D_{Ca} n_{Ca} (E/kT) / \left\{ \sum H_{iE} K_{iE} \left[q^2 D_i n_i E/kT \right] \right\} \quad [A9]$$

Ions which contribute to the total current are chlorine (Cl^-), potassium (K^+), sodium (Na^+), and calcium (Ca^{++}) ions. Considering first the flow into and out of the cell at the anodic side as shown schematically in figure A1a, we need to use the information on the ion concentration for potassium, sodium and calcium in the medium, and the concentration for that of chlorine in the cytosol. Eq. A9 can then be rewritten as:

$$J_{E_{Ca}} = (E/\rho) H_{CaE} K_{CaE} 4 e^2 D_{Ca} n_{Ca} (E/kT) / \left\{ \left[e^2 E/kT \right] \left\{ 4 H_{E_{Ca}} K_{E_{Ca}} D_{Ca} n_{Ca} + H_{E_{Na}} K_{E_{Na}} D_{Na} n_{Na} + H_{E_K} K_{E_K} D_K n_K - H_{E_{Cl}} K_{E_{Cl}} D_{Cl} n_{Cl} \right\} \right\} \quad [A10]$$

The medium was composed of 140 mM NaCl, 5.4 mM KCl, 1.5 mM MgCl², and 2 mM CaCl². The chlorine concentration in the cytosol varies between 1 and 60 mM [29]; we assumed a value of 30 mM. The data for the diffusion coefficient [16], and the partition factors [13] are listed in table 1. The hindrance factor was assumed to be 1 for all ions, since their dimensions are small compared to the pore diameter. Using the data provided in references [13, 16], we obtain

$$J_{\text{ECa}} = 0.011 (E/\rho) \quad [\text{A11}]$$

which for an E of 5.6 kV/cm gives a calcium ion current density of 0.77 A/cm² and for an E of 7.5 kV/cm a value of 1.03 A/cm².

Calculating the total calcium charge transferred into the cell through electrophoresis from the anodic side (there is no transfer out of the cell at the cathodic side, since the calcium concentration in the cytosol is zero) is obtained by solving the Eq. 3. The first variable in the integral is the calcium current density which is taken to be a constant. The second variable in the transport integral is the electroporated area through which the calcium current can flow. This area, which is schematically shown in figure A1b (indicated by the vertical line), increases with time and approaches a maximum which is dependent on the value of the applied electric field, E.

In order to express the area, A, in terms of time we use the following relations:

$$A = \pi a^2 \sin^2 \theta = \pi a^2 (1 - \cos^2 \theta) \quad [\text{A12}]$$

$$\cos \theta = V_{\text{por}} / \{f E a [1 - \exp(-t/\tau_c)]\} \quad [\text{A13}]$$

where a is the radius of the CHO cell, which is assumed to be spherical, and θ is the angle which defines the opening of the membrane as shown in figure A1 (right). Eq. A13 describes the change in θ with time, with f being a factor which depends on the shape of the cell. It is 1.5 for spherical cells. τ_c is the charging time constant of the cell and is given as [30]:

$$\tau_c = C a [\rho_s/2 + \rho_c] \quad [\text{A14}]$$

with C being the cell membrane capacitance which is generally assumed to be 1 $\mu\text{F}/\text{cm}^2$, and ρ_s and ρ_c are the resistivities of the suspension and the cytosol, respectively. In our case they are equal and expressed as ρ . V_{por} is the critical voltage for electroporation, which we assume is 1 V, although for nanosecond pulses higher values have been measured [15]. The resulting integral, with Eq. A12 and Eq. A13, can be solved analytically. The solution is:

$$Q_{\text{ECa}} = J_{\text{ECa}} \left\{ \pi a^2 (\tau + \tau_c \ln(1 - V_{\text{por}}/[fEa])) + \pi \tau_c (V_{\text{por}}/fE)^2 \{ \ln[(\exp(+\tau/\tau_c)) - 1] + 1/(1 - \exp(-\tau/\tau_c)) - \ln[V_{\text{por}}/[fEa]/(1 - V_{\text{por}}/[fEa])] + [fEa]/V_{\text{por}} \} \right\} \quad [\text{A15}]$$

Figure A2 shows the temporal development of the calcium charge in the cell when a step function electric field of 5.6 kV/cm is applied to a CHO cell with a radius of 9 μm . The characteristic time for membrane charging, τ_c , for such a cell is 108 ns. The graph shows that up to time, t_{por} , when the voltage reaches the poration voltage of 1 V, given by the condition

$$t_{\text{por}} = -\tau_c / \ln [1 - V_{\text{por}} / (fEa)] \quad [\text{A16}]$$

the calcium current is zero. It increases superlinearly when the porated area opens up (increasing θ). With increasing time the flux of calcium ions passing through the membrane approaches a constant value resulting in a linear increase in charge. The charge which is transferred into the cell from the time when the voltage is applied to the end of the pulse at 300 ns is $5.48 \cdot 10^{-13}$ As, corresponding to $1.7 \cdot 10^6$ calcium ions.

Appendix

Table A1

Diffusion coefficients and partition factors for the four dominant ion species.

Na^+	$D = 1.33 \cdot 10^{-5} \text{ cm}^2/\text{s}$	$K = 0.91$
K^+	$D = 1.96 \cdot 10^{-5} \text{ cm}^2/\text{s}$	$K = 0.91$
Cl^-	$D = 2.04 \cdot 10^{-5} \text{ cm}^2/\text{s}$	$K = 0.91$
Ca^{++}	$D = 0.79 \cdot 10^{-5} \text{ cm}^2/\text{s}$	$K = 0.83$

References

1. Tekle E, Astumian RD, Chock PB. Electroporation by Using Bipolar Oscillating Electric-Field - an Improved Method for DNA Transfection of Nih 3t3 Cells. Proc. Natl. Acad. Sci. USA. 1991; 88:4230–4234. [PubMed: 2034667]
2. Teissie J, Rols MP. An experimental evaluation of the critical potential difference inducing cell membrane electroporomeabilization. Biophys. J. 1993; 65:409–413. [PubMed: 8369446]
3. Kotnik T, Mir LM, Flisar K, Puc M, Miklavcic D. Cell membrane electroporomeabilization by symmetrical bipolar rectangular pulses. Part I. Increased efficiency of permeabilization. Bioelectrochemistry. 2001; 54:83–90. [PubMed: 11506978]
4. Kotnik T, Miklavcic D, Mir LM. Cell membrane electroporomeabilization by symmetrical bipolar rectangular pulses. Part II. Reduced electrolytic contamination. Bioelectrochemistry. 2001; 54:91–95. [PubMed: 11506979]
5. Kotnik T, Pucihar G, Rebersek M, Miklavcic D, Mir LM. Role of pulse shape in cell membrane electroporomeabilization. Biochim Biophys Acta. 2003; 1614:193–200. [PubMed: 12896812]
6. Faurie C, Rebersek M, Golzio M, Kanduser M, Escoffre J-M, Pavlin M, Teissie J, Miklavcic D, Rols M-P. Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation. J Gene Med. 2010; 12:117–125. [PubMed: 19941315]
7. Faurie C, Phez E, Golzio M, Vossen C, Lesbordes J-C, Delteil C, Teissie J, Rols M-P. Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells. BBA-Biomembranes. 2004; 1665:92–100. [PubMed: 15471575]
8. Ibey BL, Ullery JC, Pakhomova ON, Roth CC, Semenov I, Beier HT, Tarango M, Xiao S, Schoenbach KH, Pakhomov AG. Bipolar nanosecond electric pulses are less efficient at

- electropermeabilization and killing cells than monopolar pulses. *Biochemical and Biophysical Research. Communications*. 2014; 443:568–573. [PubMed: 24332942]
9. Pakhomov AG, Semenov I I, Xiao S S, Pakhomova ON, Gregory B, Schoenbach KH, Ullery JC, Beier HT, Rajulapati SR, Ibey BL. Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity. *Cell Mol Life Sci*. Apr 21.2014 [Epub ahead of print]doi:10.1007/s00018-014-1626-z.
 10. Semenov I, Xiao S, Pakhomov AG. Primary pathways of intracellular Ca^{2+} mobilization by nanosecond pulsed electric field. *Biochim. Biophys. Acta*. 2013; 1828:981–989. [PubMed: 23220180]
 11. Joshi RP, Hu Q, Schoenbach KH. Modeling studies of cell response to ultrashort, high-intensity electric fields – Implications for intracellular manipulation. *IEEE Trans. Plasma Science*. 32:1677–1686.
 12. Vasilkoski Z, Esser AT, Gowrishankar TR, Weaver JC. Membrane electroporation: The absolute rate equation and nanosecond time scale pore creation. *Phys Rev E*. 2006; 74:021904.
 13. Smith KC, Weaver JC. Transmembrane molecular transport during versus after extremely large, nanosecond electric pulses. *Biochem Biophys Res Commun*. 2011; 412:8–12. [PubMed: 21756883]
 14. Schoenbach KH, Baum CE, Joshi RP, Beebe SJ. A Scaling Law for Membrane Permeabilization with Nanopulses. *IEEE Trans. Dielect. Electr. Insul*. 2009; 16:1224–1235.
 15. Frey W, White JA, Price RO, Blackmore PF, Joshi RP, Nuccitelli R, Beebe SJ, Schoenbach KH, Kolb JF. Plasma membrane voltage changes during nanosecond pulsed electric field exposure. *Biophys. J*. 2006; 90:3608–3615. [PubMed: 16513782]
 16. DeBruin KA, Krassowska W. Modeling Electroporation in a Single Cell. II. Effects of Ionic Concentrations. *Biophys. J*. 1999; 77:1225–1233. [PubMed: 10465737]
 17. Saulis G, Venslauskas MS, Naktinis J. Kinetics of pore resealing in cell membranes after electroporation. *Bioelectrochemistry and Bioenergetics*. 1991; 26:1–13.
 18. Pavlin M, Miklavcic D. Theoretical and experimental analysis of conductivity, ion diffusion and molecular transport during electroporation – Relation between short-lived and long-lived pores. *Bioelectrochemistry*. 2008; 74:38–46. [PubMed: 18499534]
 19. He H, Chang DC, Lee Y-K. Nonlinear current response of micro electroporation and resealing dynamics for human cancer cells. *Bioelectrochemistry*. 2008; 72:161–168. [PubMed: 18314398]
 20. Tekle E, Astumian RD, Friauf WA, Chock PB. Asymmetric pore distribution and loss of membrane Lipid in electroporated DOPC vesicles. *Biophys. J*. 2001; 81:960–968. [PubMed: 11463638]
 21. Pakhomov AG, Kolb JF, White JA, Joshi RP, Xiao S, Schoenbach KH. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). *Bioelectromagnetics*. 2007; 28:655–663. [PubMed: 17654532]
 22. Benz R, Zimmermann U. The resealing process of lipid bilayers after reversible electrical breakdown. *Biochim. Biophys. Acta*. 1981; 640:169–178. [PubMed: 7213683]
 23. Puc M, Kotmik T, Mir LM, Miklavcic D. Quantitative model of small molecules uptake after in vitro cell electropermeabilization. *Bioelectrochemistry*. 2003; 60:1–10. [PubMed: 12893304]
 24. Al-Baldawi NF, Abercrombie RF. Calcium diffusion coefficient in *Myxicola* axoplasm. *Cell Calcium*. 1995; 17:422–430. [PubMed: 8521456]
 25. Li J, Lin H. Numerical simulation of molecular uptake via electroporation. *Bioelectrochemistry*. 2011; 82:10–21. [PubMed: 21621484]
 26. Ibey BL, Xiao S, Schoenbach KH, Murphy MR, Pakhomov AG. Plasma membrane permeabilization by 60- and 600–ns electrical pulses is determined by the absorbed dose. *Bioelectromagnetics*. 2009; 30:92–99. [PubMed: 18839412]
 27. Saulis, G. Kinetics of pore formation and disappearance in the cell during electroporation. In: Pakhomov, AG.; Miklavcic, D.; Markov, MS., editors. *Advanced Electroporation Techniques in Biology in Medicine*. CRC Press; Boca Raton: 2010. p. 213-238.
 28. Foster KR, Bidinger JM, Carpenter DO. The electrical resistivity of cytoplasm. *Biophys. J*. 1976; 16:991–1001. [PubMed: 963211]
 29. Molleman, A. *Electrophysiology*. John Wiley & Sons, Ltd; Chichester, Great Britain: 2003. Patch Clamping - An Introductory Guide to Patch Clamp. table 2.1

30. Cole KS. Electrical impedance of marine egg membranes. *Trans. Faraday Soc.* 1937; 23:966–972.

Highlights

- A mechanism for reduced ion uptake with bipolar pulses is proposed.
- The mechanism relies on the concept of reverse electrophoresis.
- Delaying opposite polarity pulse shifts uptake from electrophoresis to diffusion.
- Pore recovery time is obtained by comparing monopolar with bipolar pulse effects.
- Result indicate short (microsecond) pore recovery times.

Fig. 1.

Insert: Pulse shapes of the 300 ns monopolar pulse and the 600 ns bipolar pulse which were applied to CHO cells. In addition, bipolar pulses, where the second pulse of opposite polarity followed the first pulse up to 10 μs later, were also used in this study. Histogram: The maximum measured calcium concentration in the CHO cells is shown for two electric field intensities: 5.6 and 7.5 kV/cm. The experiments were performed with 2 mM of extracellular calcium after its depletion from the endoplasmic reticulum with CPA. The hatched and white bars correspond to the monopolar and bipolar pulses, respectively. The results obtained with delayed secondary pulses are shown for pulse separations of 0.4, 1, 5, and 10 μs .

Fig. 2.

Left: Shape of the 60 ns monopolar and bipolar pulses. Right: Histogram, showing the maximum measured cytosolic calcium concentration for two electric field intensities, 26 kV/cm and 30 kV/cm. Hatched bar: results obtained with a single monopolar pulse of 60 ns duration. White bar: results obtained with a single bipolar pulse. The experiments were performed with 2 mM of extracellular calcium after its depletion from the endoplasmic reticulum with CPA.

Fig. 3.

Schematic illustration of the flow pattern and the calcium concentration pattern, before, during and after the pulses. The black circle with the light grey ring depicts the cell membrane, the darker grey areas the range of high calcium ion concentration. With the first pulse generating nanopores at the cell membrane, which allow the flow of light ions into the cell, calcium ions at the anode side of the cell (left) are drifting into the cell, and at the cathode (right), away from the cell. This is shown as a shift of the range of high calcium concentration in cathode direction. When the second pulse with inverse polarity is applied, calcium ions flow in the opposite direction, which leads to an almost complete re-establishing of the initial conditions. After the pulses are applied, diffusion becomes the major ion transport mechanism. Since this is a transport process which is determined by the density gradient, it causes a radial flow into the cell, which results in an increasing layer of calcium ions inside the cell, as depicted by the red circle. This process, which can be neglected for the time of pulsing, as shown in the supporting information, continues as long as the nanopores are open.

Fig. 4.

Schematic illustration of the diffusion processes close to the porated membrane with thickness, d , at the anode side (left) and the cathode side (right) after a monopolar pulse is applied. a) At the anode side, the calcium ions diffuse from the electrophoresis-generated layer of high concentration, which has a width of the electrophoresis length, L_E , at the inside of the cell, towards the center of the cell. At the cathode side the layer of depleted calcium ion concentration outside the cell – also with a thickness of L_E – is filled up through diffusion. b) The graphs in the bottom part of figure 4. show schematically the temporal development of the calcium ion concentration in the anode (left) and cathode (right) region with increasing time ($t_0 < t_1 < t_2$).

Fig. 5.

Temporal development of the membrane conductance during the pore resealing process. Data for the long recovery time curve (dotted line with measured conductance values) were obtained by means of patch clamp measurements on GH3 cells [21]. It shows the recovery of the membrane after application of a single 60 ns pulse at 12 kV/cm. Data for the fast recovery (solid line) were obtained by a pulse - probe method (first pulse served to electroporate the membrane, second pulse to measure the conductance) applied to black lipid bilayer membranes [22]. Shortest pulses applied in this study had a duration of 300 ns. Recovery curves obtained from our modeling approach are shown as long dashed (300 ns, 5.6 kV/cm) and short dashed lines (300 ns, 7.5 kV/cm).