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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 those 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 10 μs.

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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 μs pulse duration for a total pulse train duration of 400 μs. The conclusion of these studies was that bipolar pulses have a positive effect on electropermeabilization compared to monopolar pulses. The reason given was generally that bipolar pulses compensate for the asymmetry of electropermeabilization 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 electropermeabilization 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 μM at 5.6 kV/cm. It increased to 3.8 μM 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 μs, 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

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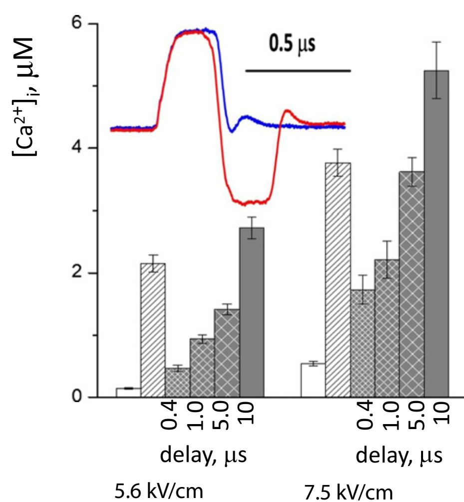

Fig. 1. Inset: Pulse shapes of the 300 ns monopolar pulse and the 600 ns bipolar pulse which were applied to CHO cells. In addition, bipolar pulses, where the second pulse of opposite polarity followed the first pulse up to 10 μs later, were also used in this study. Histogram: The maximum measured calcium 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.

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, inset), 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 integral 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 Eq. (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/cm^2 and for an electric field intensity of 7.5 kV/cm the value is 1.03 A/cm^2.

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

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