Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 282-287

ELECTRO-PERMEABILIZATION OF CELL MEMBRANES : EFFECT OF THE RESTING MEMBRANE POTENTIAL

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Received August 9, 1990

Summary: Electric field induced permeabilization of cell membranes is an important technique for gene transfection and cell hybridization[1-4]. Mechanistic studies of this process revealed that the *uptake* of fluorescent indicator by plant protoplasts occurs predominantly on the hemisphere facing the positive electrode[5], while in erythrocyte ghosts the probes *exit* through the hemisphere facing the negative electrode[6]. To reconcile these observations symmetrical pore formation and a mechanism of molecular exchange by electroosmosis has been proposed[6]. In light of these controversial observations, we conducted a systematic study of electroporation of NIH3T3 cells with varying electric field strength, waveform and frequency. Our data revealed that (i) symmetrical permeabilization of the cell membrane occurs only with bipolar a.c. fields. (ii) When a critical membrane breakdown potential, V_c, is applied using either an unipolar a.c. fields or a single d.c. square pulse, the cell membrane becomes permeabilized only at the hemisphere facing the positive electrode. (iii) When the pulse - induced membrane potential, V_m, is approximately equal to or larger than the intrinsic membrane potential (i.e. using d.c. or unipolar a.c. field), asymmetric permeabilization was observed with the hemisphere facing the positive electrode being most permeable. (iv) The rate of fluorescent indicator uptake is dependent on the concentration of the indicator. These results indicate that electro-permeabilization of cell membranes is affected by its resting potential and that electroosmosis is not the dominant mechanism for the cellular uptake of foreign molecules in electroporation.

Electric field of sufficient magnitude and duration has been shown to create transient pores in cellular membranes. This method has been widely used for introducing DNAs, allosteric effectors, antibodies and other macromolecules into living cells either by molecular exchange between intra and extracellular media or by cell to cell fusion. In many instances, these field methods have proved to be superior to the more conventional methods of chemical or viral - induced cell transfection and fusion. Some studies further suggest that electric fields could be used to modulate the activities of membrane enzymes in transducing biological signal and energy[7]. In spite of these advances, however, mechanisms by which the electric field induces permeability or fusion among cells is largely unknown. In this study, various amplitudes of unipolar and bipolar oscillating, as well as single d.c. electric fields, was applied to NIH3T3 cells in order to learn about the location and lifetime of electric field- induced pores. Through time course measurements, we further follow the process of diffusion of a marker probe molecule into the cell interior.

Most biological membranes *in vivo* maintain a resting membrane potential difference which may range from about -30 to -180 mV. An applied field causes an induced membrane potential which

0006-291X/90 $1.50

adds vectorially to this. Because the lipid bilayer is several orders of magnitude less conductive than the aqueous medium, ions of opposite signs accumulate at the interior and exterior surfaces. Consequently, an applied field is greatly magnified (with the magnification proportional to the the radius of the cell) within the membrane. For an oscillating electric field, the frequency dependence of the induced membrane potential is given by [1]

$$V_m = \frac{3/2 \; R \; E}{\sqrt{1 + (2\pi f \tau)^2}} \cos(\theta) \qquad (1)$$

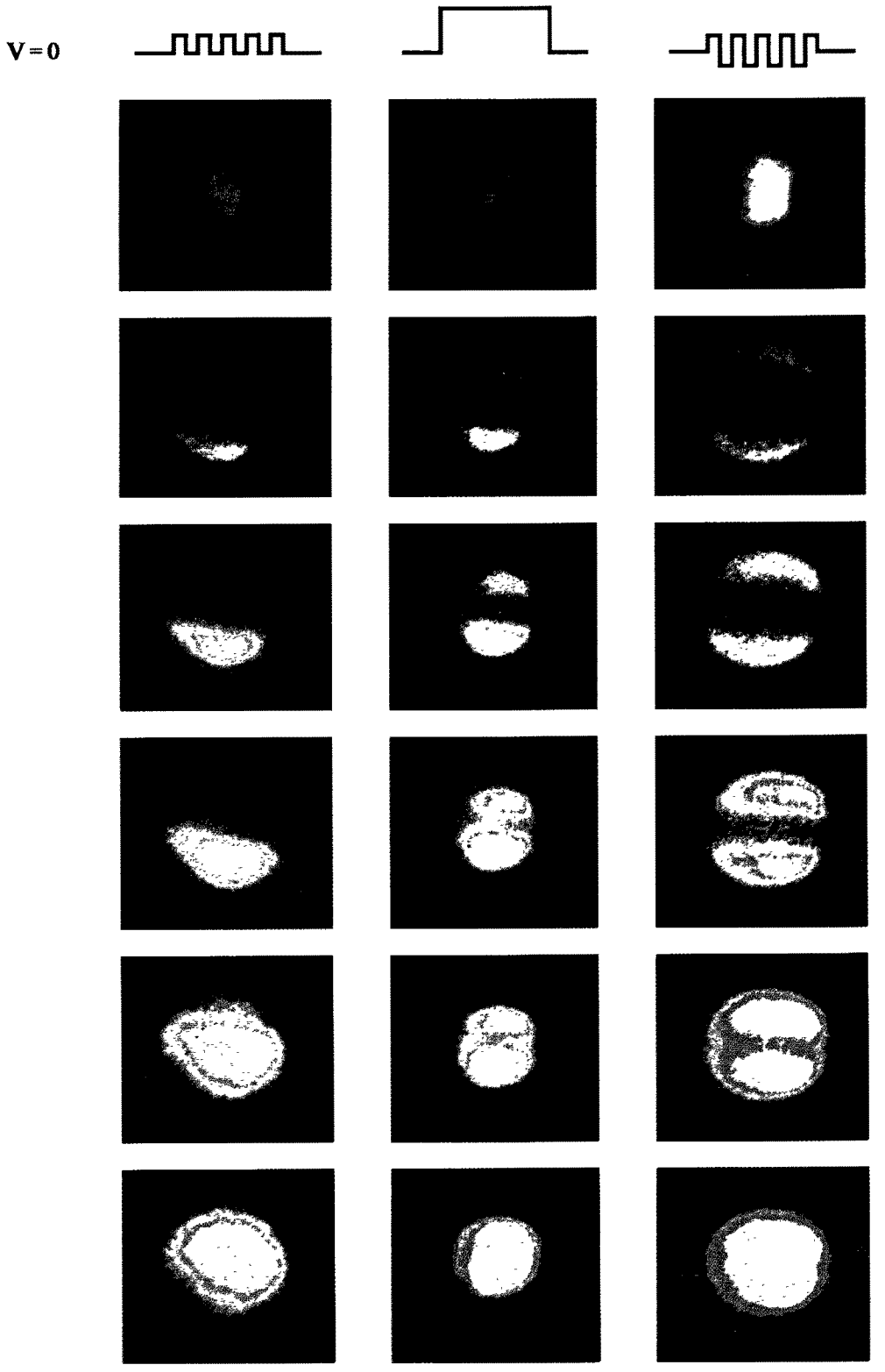
where τ is the relaxation time, f the frequency of the applied field, R the radius of the cell, E the electric field strength, and θ is the angle between the field direction and any point on the cell membrane. For d.c. fields, f=0 so that the denominator of eq.(1) is reduced to 1. If the total imposed membrane potential (i.e. that due to the field coupled to the resting potential) reaches some critical value, electrical breakdown occurs and it becomes permeable to otherwise impermeable ions and molecules. Equation (1) shows that the maximum electrical effect would be at the two poles of the cell membrane facing the electrodes (i.e. $\cos(\theta) = 1$ for $\theta = 0$, and -1 for $\theta = \pi$). In this study we demonstrate that membrane breakdown is essentially asymmetric with unipolar a.c. or single d.c. electric fields. Symmetrical permeabilization occurs only with bipolar a.c. fields. Our results indicate that resting membrane potential plays a role in electro-permeabilization of the cell membrane and electroosmosis is not the dominant mechanism in the cellular uptake of molecules in electroporation. In addition, taking the advantages of bipolar a.c. fields, we have shown that one can obtain a significant improvement in the efficiency of DNA transfection using a bipolar a.c. field electroporation technique.

Materials and methods

Wild type NIH3T3 cells were grown to approximately 50 -70 % confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % calf serum. They were then removed with 0.25 % trypsin solution and washed three times in the pulsing buffer solution which consists of 250 mM sucrose, 10 mM phosphate, and 1 mM $MgCl_2$, pH 7.2. The viability of cells suspended in the pulsing buffer was not altered for up to 2 hrs. For the pulse experiments, 0.050 ml of the cell suspension was mixed with a stock solution of ethidium bromide (final concentration = 0.01 %) and a portion is rapidly transferred to the electrode chamber sandwitched between two microscope slides. All experiments were done within five minutes after mixing of the dye. The electrode chamber was mounted on a ZEISS (ICM405) inverted low light fluorescence microscope. The electric pulses of desired amplitude and frequency were supplied from two pulse generators (Cober 606P). Real time images of electro-permeabilization events were acquired with an image intensifier (Videoscope international, KS-1381) attached to a CCD camera (COHU, 4815) and recorded on videotape (Sony, VO-5600). The recorded images were later transfered to a digital disc recorder (Panasonic, TQ-2028F) and processed with a digital image processor (Recognition Concepts, 55/48Q) using the RTIPS software library from TAU Inc. Selected video images were then displayed on a color monitor and photographed by transfering to a freeze frame recorder (Polaroid Corp.).

Results

Fig.1 depicts selected video frames of both the location and time course of electro-permeabilized NIH3T3 cells subjected to various waveforms of different amplitude and frequency (see Fig. caption). Ethidium bromide was used as a permeabilization indicator probe. Its fluorescent intensity monitored at 610 nm (excited at 520 nm) is enhanced drastically when it binds to DNA or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RNA. In the absence of an applied electric field, ethidium bromide can diffuse slowly into the NIH3T3 cell which is responsible for revealing the nucleus of the cells shown in the first row (Fig.1). Typically, the cells would be stained completely if they were incubated with the indicator under the experimental conditions for 20 to 30 minutes. Therefore, all experiments were performed within 5 minutes after the mixing of the dye. Attempts to observe the exit of the ethidium bromide-nucleic acid complexes in fully stained cells were not successful. This could be due either to the size of the electropores being too small for the complexes or the rate too slow to permit detection. We have, however, observed (data not shown) that under intense electric fields (for example, 4 kV/cm) the fluorescence in the fully stained cells gradually disappears in about 1 minute, giving rise to a small increase in the background fluorescence.

The left column in Fig.1 shows results from an unipolar a.c. field with a frequency of 250 KHz and electric field magnitude of 1.1 kV/cm, which is near the field strength required for membrane breakdown (about 0.8 - 1.0 kV/cm). No qualitative difference was observed in the experiments between unipolar a.c. and single d.c. pulses. The data clearly show that only the hemisphere of the cell membrane facing the positive electrode is permeabilized. It should be pointed out that some of the larger cells can be permeabilized with only 0.8 kV/cm. When the applied electric field is gradually increased, often by about 100 to 300 V/cm, one also observes the entry of the fluorescent probe, at a much reduced quantity, from the hemisphere facing the negative electrode. The additional voltage required to initiate the second entry site can be attributed to the field strength needed to overcome the resting membrane potential, which is calculated from eq.(1) to be in the range of -80 to -210 mV. Although the entry positions on the cell surface remain unchanged, the permeabilized area increases with increasing electric field. For example, when the applied electric field is greatly enhanced, significant permeabilization is observed at both hemispheres facing the electrodes. The permeabilization, however, remains asymmetric with the effect being more pronounced at the site facing the positive electrode than the other. This was demonstrated using either d.c. or unipolar a.c. fields. The results from a 0.400 msec time window, 4.95 kV/cm single d.c. field is shown in the middle column of frames in Fig.1. Under these conditions, the first available frame after the pulse (33 ms) show that the asymmetric permeabilization occurs simultaneously on both sides (data not shown). On the right column of frames in Fig.1, we show that, unlike those observed with either d.c. or unipolar a.c. fields, the bipolar a.c. field induces symmetric permeabilization of the cell membrane. All experiments reported above were also performed using wild type Chinese Hamster Ovary (CHO) cells and similar results were obtained. To establish the utility of the bipolar a.c. permeabilization method, we carried out transfection experiments on NIH3T3 cells using a plasmid DNA, pSV2-neo (we wish to thank Dr. S.G. Rhee for providing

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Fig.1 Selected videoframes of real time electropermeabiliztion events in NIH3T3 cells subjected to electric fields of various waveforms. The positive electrode is at the bottom and the negative electrode is at the top of each frame displayed. The first row (black & white) for each waveform shows the prepulse image where the nucleus is relatively bright due to the slow uptake of ethidium bromide during sample preparation. In subsequent frames showing events after the pulse, the prepulse image has been subtracted. The fluorescence is color coded in increasing intensity in the following order: Blue, purple, gray, yellow, red, and white. For each waveform displayed, time increases from top to bottom frame. Pulse duration = 0.400 msec. From left to right, i) Unipolar a.c. pulse: Electric field, E = 1.10 kV/cm, Frequency = 250 KHz. Time series of frames : 0.528, 1.508, 2.739, 6.303, 8.745 seconds. ii) d.c. square pulse: E = 4.95 kV/cm. Time series of frames: 0.297, 0.825, 1.419, 2.013, 8.948 seconds. iii) Bipolar a.c. pulse: E = 2.25 kV/cm (peak to peak). Frequency = 250 KHz. Time series of frames: 0.132, 0.396, 0.891, 1.848, 3.267 seconds.

this plasmid.). The plasmid contains a neomycin resistant gene which if integrated into the cellular genome can be used to select transformed cells. Comparative experiments showed that the bipolar a.c. field results in an improved transfection efficiency (i.e. transformants/μg DNA) per 10^5 plated cells by as much as as 1.7 and 5.5 fold over the the unipolar a.c. and single d.c. square pulses respectively (data not shown, manuscript in preparation).

Discussion

The results reported here are consistent with the hypothesis that the negative resting potential interacts vectorially with the imposed electric potential. A negative resting potential means that the equilibrium electric field in the membrane is directed inwards. When an external electric field is applied, the sign of the imposed membrane potential is (+) inside and (-) outside on the pole of the cell membrane facing the negative electrode and the opposite is true on the other pole facing the positive electrode. Thus, when the applied electric field alone is nearly equal to or slightly greater than that required for membrane breakdown, the field vectors are additive and greater than V_C at the membrane site facing the positive electrode, while they cancel on the membrane site facing the negative electrode and resulted with a net potential being less than V_C. Once the applied potential overcomes this cancellation, a second permeabilized site is observed at the hemisphere facing the negative electrode. The fact that ethidium bromide is able to enter from both hemispheres at high electric fields suggests that electrophoretic or electroosmotic mechanisms cannot be the predominant factors since both processes are fundamentally unidirectional. The observed asymmetrical entry of the fluorescent probe with high field could be due to the fact that the fluorescence indicator is positively charged. When a d.c. or unipolar a.c. field is applied, the indicator molecules migrate towards the negative electrode. Consequently, their population would be higher near the hemisphere facing the positive electrode but lower at the hemisphere facing the negative electrode. It should be pointed out that quantitatively, the effect of the imposed electric field is also dependent on the size and age of the cells used and the possible inhomogeniety of the applied electric field. Additionally, we have observed strong concentration dependence with respect to the rate of ethidium bromide uptake by the cells under the same applied electric field (data not shown). This observation suggests that diffusion as a likely molecular exchange process instead of electropheresis or electroosmosis

Together with the qualitative results presented above, the permeabilization of the membrane at multiple sites without significantly affecting cell serviability may account for the observed improvements in transfection efficiency (i.e. the number of cells transformed per μg of DNA used) of electro-permeabilized NIH3T3 cells with bipolar a.c. fields compared to single d.c. or unipolar a.c. fields . We expect that the bipolar a.c.field method would also exhibit similar advantages when applied to electrofusion experiments where contact of permeabilized areas of different cells is an important factor for successful fusion .

Acknowledgments

We thank Dr Kenneth Spring for his generosity in helping with instrumentation and image analysis and Dr. Blair Bower for the use of her fluorescent microscope setup. One of us (E.T.) is a recipient of the National Research Council research associateship.

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