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Electroporation by using bipolar oscillating electric field: An improved method for DNA transfection of NIH 3T3 cells

(gene transfection/electro-permeabilization)

EPHREM TEKLE*, R. DEAN ASTUMIAN†, AND P. BOON CHOCK*

*Section on Metabolic Regulation, Laboratory of Biochemistry/National Heart, Lung and Blood Institute, National Institutes of Health, Building 3, Room 203, Bethesda, MD 20892; and †Biotechnology Division, National Institute of Standards and Technology, Building 230, Room 105, Gaithersburg, MD 20899

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ABSTRACT **Using the plasmid DNA pSV2-neo (which, when integrated into the cellular genome confers resistance to the antibiotic G418 for selection), we examined and compared the transfection efficiency on NIH 3T3 cells electro-permeabilized by applying a sequence of high-frequency unipolar or bipolar square waves or a single square pulse. Results show that a bipolar square wave is, at least, 1.7- and 5.5-fold more efficient than the unipolar square wave and single square pulse, respectively. In the range of electric field strength used for optimum transfection, the survivability of electro-permeabilized cells was comparable between the unipolar and bipolar square waves but fell considerably with the single square pulse. Qualitative comparison of cell permeabilization induced by the three types of wave forms and monitored by ethidium bromide uptake revealed that only the bipolar square wave permeabilizes the cell membrane symmetrically at the two hemispheres facing the electrodes. With unipolar square wave or single square pulse, the membrane is permeabilized either on one side or asymmetrically. Taken together, our result suggests that permeabilization of the membrane at multiple sites without affecting cell survivability may account for the improvements in transfection efficiency observed with bipolar oscillating electric fields.**

Cells in suspension or in culture that are subjected to some critical electric field strength and duration are transiently permeabilized (for reviews, see refs. 1–5). This technique allows one to introduce exogenous DNAs and other macromolecules into cells. Often, this field method has proved superior (6, 7) to the more conventional methods (8, 9) of transfection. Since the demonstration by Neumann *et al.* (10, 11) on the uptake and expression of a plasmid DNA in cultured mouse fibroblast cells by electro-permeabilization, additional work has been reported with other mammalian (10–13), bacterial (6, 14), yeast (15), and plant (16, 17) cells. In recent years, attention has focused toward improving and optimizing the transfection efficiency by manipulating various experimental parameters (10, 12, 18–21). However, manipulation of the electrical variables (i.e., field strength, pulse duration, frequency, and wave form) to improve gene transfections have not, as yet, been fully exploited. In a recent report, a dc-shifted radio frequency ac pulse was more efficient than a single square pulse of similar amplitude and duration (22). Unlike previous techniques that have used unipolar wave forms (capacitor-discharged exponential decay pulses, single square pulses, or dc-shifted radio frequency ac pulses), we introduce a relatively efficient method of electro-permeabilization by using high-frequency bipolar oscillating electric pulses. The bipolar field induces symmetrical permeabilization of the membrane, whereas the unipolar-wave-form permeabilization is found to be either one sided or asymmetrical.

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MATERIALS AND METHODS

Electric Pulse Apparatus. We have assembled an electric pulse apparatus capable of producing several types of wave forms with frequencies up to 1 MHz. The block diagram of the instrument arranged to generate bipolar oscillating electric pulses is shown in Fig. 1*a*. Duration of the bipolar square wave burst is controlled by a single square-pulse generator (Hewlett–Packard model 8011A) the output of which gates the pulse-frequency generator (Wavetek model 183). The rising edge of each single square pulse from the pulse-frequency generator triggers the high-power positive pulse generator (Cober model 606P). Amplitude and width of the pulse from the Cober generator are independently adjustable from 0 to +2.5 kV and from 50 ns to 10 ms, respectively. The falling edge of a square pulse from the Cober generator is then allowed to trigger the second high-power pulse generator (Cober model 606P) with similar adjustable output (but with amplitude of 0 to −2.5 kV). Combination of these pulses finally produces the desired bipolar square wave form with peak-to-peak amplitude of up to 5 kV. The maximum delay in the trigger mechanism between the two high-power generators is <0.5 μs. The magnitudes of the electric pulses were measured across the electrodes with a 1000:1 probe (Tektronix model P6015) and monitored on a storage oscilloscope (Tektronix model 7704A). Single square pulses of either polarity were produced by disabling the pulse-frequency generator and one of the high-power pulse generators. Similarly, unipolar oscillating pulses were obtained by disabling any one of the high-power generators, depending on the polarity desired. Typical examples of the wave forms obtained with the present instrument measured across a 500-Ω load resistor are shown in Fig. 1*b*. This setup also allows one to obtain other wave forms. The electrodes consisted of two 50-mm-long stainless steel plates and were held fixed on a Kel-F (3M Co.) support in grooves with a separation of either 1 or 2 mm. A culture dish containing cells grown in monolayer or suspended in droplets was placed on a platform that could be moved vertically to contact the electrodes. In addition, horizontal platform movement allows up to 12 experiments to be done on the same culture dish (8-cm diameter).

Fluorescence Microscope and Image Analysis. An electrode chamber sandwiched between two microscope slides (electrode separation, 0.75 mm) with a capacity to hold 25 μl of cell suspension was constructed. The chamber was mounted on a Zeiss model ICM405 inverted low-light fluorescence microscope. The increase in fluorescence intensity, monitored at 610 nm with excitation wavelength set at 520 nm, that results from ethidium bromide (Sigma) binding to cytosolic DNA or RNA, was used to monitor electro-permeabilization. Real-time images of these events were acquired with an image intensifier (Videoscope International model KS-1381)

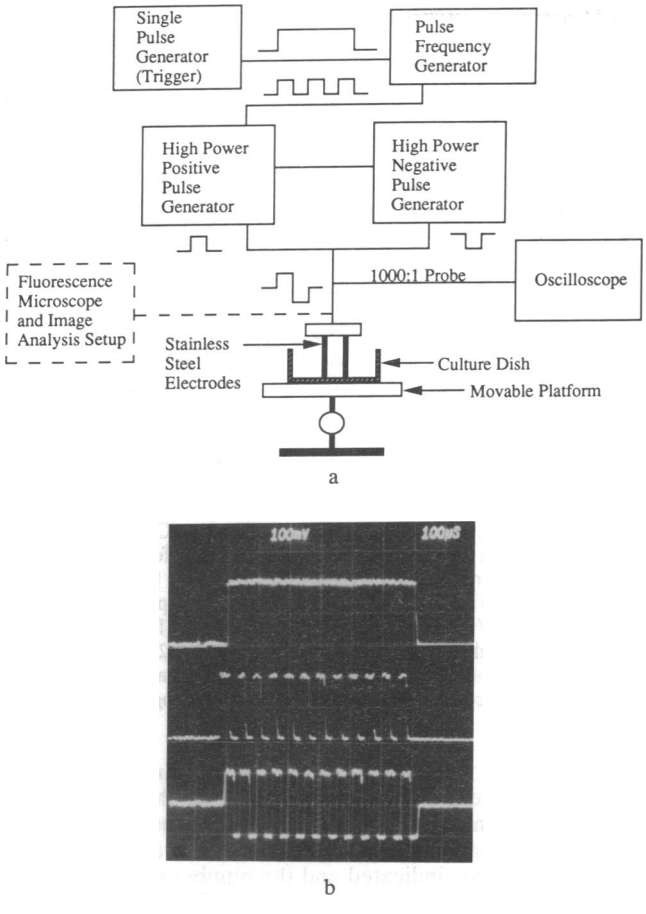

FIG. 1. (*a*) Block diagram of electro-permeabilization apparatus, showing connections arranged to produce bipolar pulses. The dotted block represents attachments of the instrument to a fluorescence microscope and image analysis setup with a separate electrode chamber. (*b*) Oscilloscope traces of the different wave forms used. Abscissa is 100 μs per division, and ordinate is 100 mV per division.

attached to a charge-coupled device (CCD) camera (Cohu model 4815) and recorded (Sony model VO-5600) on a videotape; the time resolution of the videoscope recordings was 33 ms. The recorded images were later transferred to a digital disk recorder (Panasonic model TQ2028F) and analyzed with a digital image processor (Recognition Concepts model 55/48Q) by using the RTIPS software library from Tau (Los Gatos, CA). Selected images in the time series were then displayed on a color monitor and photographed by transferring to a freeze-frame recorder (Polaroid).

Cells, Media, and Plasmid. NIH 3T3 cells (average radius, 15 μm, wild type and mutants lacking the epidermal growth receptor) were grown to ≈50–70% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Adherent cells were removed with 0.25% trypsin solution and washed three times with a pulsing buffer consisting of 250 mM sucrose, 10 mM phosphate, 1 mM $MgCl_2$, pH 7.2. The number of harvested cells was determined in at least three readings by using an hemocytometer chamber. Cells were kept at ice temperature before both DNA transfection and permeabilization experiments. For experiments on cells adherent to the culture dish, the medium was replaced with cold pulsing buffer. The viability of cells suspended in the pulsing buffer was not altered for up to 2 hr. The circular plasmid DNA pSV2-neo (≈8 kb) was provided by S. G. Rhee of our laboratory. Powdered antibiotic G418 sulfate was obtained from GIBCO; its specific activity was 516.7 μg per mg of powder. The stock solution of selection medium contained 400 μg of active antibiotic per ml of growth medium.

Transfection, Permeabilization, and Cell Survival. All transfection experiments were done typically within ≈20 min of harvesting fresh cells. Ten minutes before applying an electric pulse, an appropriate concentration of plasmid DNA was added to a suspension of cells (10^6 cells per ml) in the pulsing buffer and incubated at ice-water temperature. An aliquot of the homogeneous cell suspension (100 μl) was carefully placed as a thin strip on an empty culture dish mounted on the movable platform (Fig. 1*a*). After proper alignment, the platform was moved vertically toward the fixed parallel electrodes and, upon contact, the strip of cell suspension was evenly distributed between the electrodes through capillary action. The electrodes were sterilized with absolute alcohol and blow-dried before each use. After application of the electric pulse of desired amplitude and duration, the culture dish was immediately removed, covered, and placed on ice-water temperature for 25–30 min. Cells were then diluted with 5 ml of growth medium on the same culture dish and distributed in a 96-well flat bed chamber (Costar) at 10^3 cells per well and kept in the incubator for 6–8 hr. Afterward, the medium was aspirated off and replaced with the selection medium at 100 μl per well. Every 2 days, the selection medium was replaced with fresh medium, and after 14 days the number of G418 resistant colonies was counted in each well. Several parallel control experiments were done with the same protocol omitting either plasmid or electric field or both. Permeabilized sites on the membrane were identified via the increase in fluorescence intensity resulting from binding of ethidium bromide to cytosolic DNA/RNA; concentration of ethidium bromide ranged from 0.01% to 1%. Reversibility of the permeabilized cells was monitored by using trypan blue stain (GIBCO; final concentration, 0.04%) exclusion method as a function of time. Survivability of cells was assayed by using trypan blue introduced 1 hr after termination of the electric pulses; survivability given is an average of at least three determinations.

RESULTS

The relative efficiency of DNA transfection of many cell types may depend on a multitude of parameters (i.e., temperature, form of DNA, ionic strength, etc.). However, the critical parameters have generally been the electric field strength and pulse duration. We will, thus, focus on the effects of these two parameters. To minimize ohmic heating, we used a low-ionic-strength pulsing buffer. For most field strengths and durations used, temperature will rise by <0.2°C. In addition, the number of cells subjected to inhomogeneous field at the edges of the electrodes is negligible compared with those between the parallel (5 cm) electrodes.

Cell Survival and Transient Permeabilization. Fig. 2 shows the effect of electric field on cells grown in a culture dish. The dark thin stripes show cells, which lay between the two parallel electrodes, that have been stained with trypan blue. For qualitative identification of the electrical parameters, this procedure is also quick and simple with minimal disturbance to the adherent cells before electric field application. Based on this procedure, cell survival was examined as a function of electrical parameters, and some results are summarized in Table 1. Viability (percent survival) was quantitated 1 hr after an electric pulse with the trypan blue method. We found no further improvements in viability for cells incubated for longer than 1 hr. Note that trypan blue has been widely used to measure survivability of cells. However, recently Weaver *et al.* (23) showed that the dye-exclusion method may not reflect the survivability of *Schizosaccharomyces pombe* cells after electro-permeabilization. For all three wave forms the viability of cells was found to decrease with increased electric

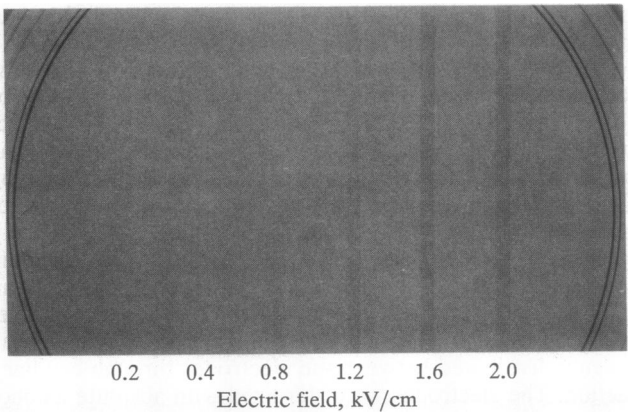

FIG. 2. Results of a typical electro-permeabilization experiment done on adherent cells on a culture dish. Dark stripes show trypan blue-stained cells that lay between two electrodes. The dye was introduced 1 hr after termination of the electric pulse, and labeling was continued for 30 min, after which the cells were washed and photographed against a white background. The wave form used was a single square pulse, and pulse duration was 400 μs.

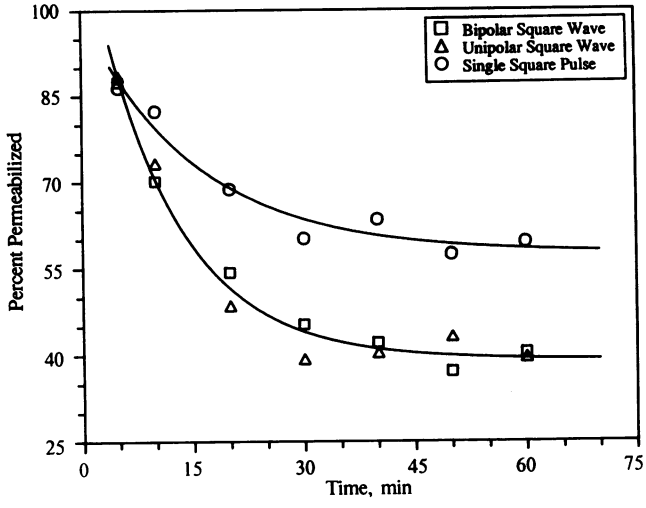

FIG. 3. Reversible electro-permeabilization. Cells were permeabilized by using the optimum field strength found for transfection for each wave form. Pulse duration was 400 μs; frequency (when applied) was 60 kHz. □, Bipolar square wave (peak-to-peak field strength, 2.2 kV/cm); △, unipolar square wave (field strength, 1.6 kV/cm); ○, single square pulse (field strength, 1.2 kV/cm). Trypan blue was added after the electric field application at the time shown. Permeabilized cells were measured by counting the number of stained cells.

field, but the survivability was higher with oscillating pulses than with a single square pulse for a given field strength. For field strengths below ≈1.6 kV/cm and a frequency of 60 kHz, increasing pulse duration from 100 μs to 1 ms did not appreciably change cell viability. Increasing pulse duration to 5 ms and above (field strength 1.6 kV/cm), however, sharply increased cell death (Table 1). The frequency dependence of cell viability showed the opposite effect. At 1.6 kV/cm, for example, cells treated with the unipolar pulse recovered by ≈20% when frequency was raised from 60 kHz to 1 MHz (Table 1). Similar recovery was also obtained with the bipolar pulse when the electric field was fixed at 2.5 kV/cm (data not shown). However, when other parameters are maintained fairly constant, increases in frequency from 20 kHz to 80 kHz did not appreciably affect cell viability for the pulse duration of 400 μs used in these experiments (data not shown).

When applied field strength and pulse duration are in the moderate range, permeabilization of the cell membrane is reversible. Fig. 3 depicts the time courses for resealing of the permeabilized membrane caused by the three wave forms studied. For each wave form, we used the electric field strength at which optimum transfection was observed (see Table 1). Trypan blue was added to the cells after the electric pulse at the time indicated and the number of cells incorporating the dye were counted. The data fit reasonably well to an exponential function. Extrapolation of the curves to the y axis shows that almost all surviving cells are permeabilized at these field strengths. Time course for the bipolar and unipolar square wave-treated cells extrapolated to ≈110% permeabilization, which may indicate that, initially, the recovery is preceded with a lag time. The data also show that maximal recovery is achieved within 30–35 min.

DNA Transfections. Data in Table 1 show that the relative transfection efficiency is a function of the field strength, wave

Table 1. Summary of permeabilization and transfection efficiency as a function of various parameters

Electric field,* kV/cm	Frequency,† kHz	Pulse duration, μs	NCBEP $\times 10^{-5}$	Plasmid added, μg/ml	Survival, %			Efficiency, transformants per μg		
					BSW	USW	SSP	BSW	USW	SSP
0.4	60	400	1.1	10	97	95	95	15	19	14
0.8	60	400	1.2	10	93	95	81	29	21	20
1.2	60	400	1.1	10	93	77	43	13	213	129
1.6	60	400	1.2	10	89	59	27	108	417	87
2.2	60	400	1.2	10	62	25	17	710	191	22
3.0	60	400	1.1	10	44	11	7	229	39	3
4.0	60	400	1.0	10	19	3	<1	15	2	0
1.6	60	400	1.1	1				30	70	10
1.6	60	400	1.2	5				98	392	94
1.6	60	400	1.1	20				115	408	96
1.6	60	1000	1.0	10	81	53	26	103	359	71
1.6	60	5000	1.1	10	41	28	21	48	92	14
1.6	250	400	1.0	5	93	68		22	112	
1.6	1000‡	400	1.2	5	92	78		16	48	
0	0	0	1.2	10	96	96	98	0	0	0
1.6	60	400	1.1	0	86	61	38	0	0	0
0	0	0	1.0	0	95	98	96	0	0	0

NCBEP, number of cells before electric pulse; BSW, bipolar square wave; USW, unipolar square wave; SSP, single square pulse.
*Electric field for BSW is evaluated from the peak-to-peak voltage; therefore, cells receive half of this field strength for any given time.
†These data apply only to BSW and USW.
‡Due to dead time (0.5 μs) in the trigger mechanism between the two Cober generators, BSW may be distorted at this frequency.

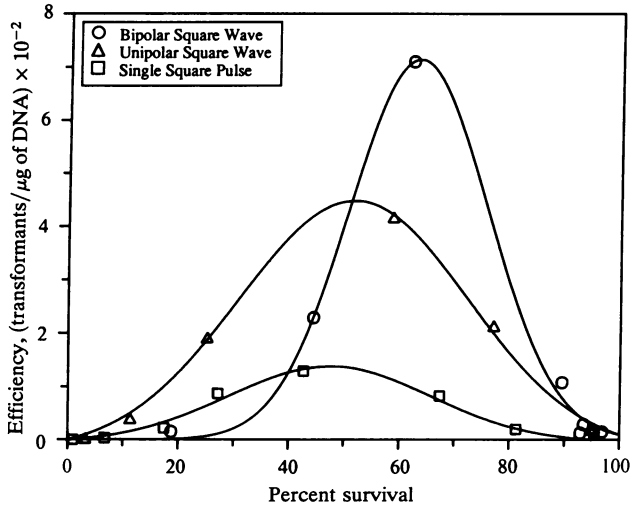

FIG. 4. Relationship between transfection efficiency and percent survival. Data were obtained with 60 kHz (when applied), 400-μs pulse duration, and field strength varying from 0.4 to 4 kV/cm. Initially the number of cells present were ≈1.1 × 10^5 with plasmid at 10 μg/ml.

form, frequency, and pulse duration. The data represent the average of two independent experiments; mean deviation was <7%. The optimum transfection was found at electric field strength of 1.2, 1.6, and 2.2 kV/cm for single square pulse, unipolar square wave, and bipolar square wave, respectively. Under these optimal conditions for each wave form, the transfection efficiency with the bipolar square wave pulse was 1.7- and 5.5-fold greater than the unipolar square wave and single square pulse, respectively. Note that the values given for transfection efficiency in Table 1 are relative numbers. Other parameters, such as ionic strength, divalent ion concentration, etc., can also affect transfection efficiency. Fig. 4 shows the relationship between transfection efficiency and percent survivability. It is interesting to note that a maximum is obtained for each wave form applied. To ensure that these comparative experiments were not done at limiting or saturating levels of the plasmid DNA, transfection efficiency was studied as a function of plasmid concentration (Table 1). The number of transformants was found to increase linearly when plasmid concentrations varied from 5 μg/ml to 20 μg/ml. The efficiency remained relatively constant over this concentration range, but decreased sharply for all wave forms when plasmid concentration was lowered to 1 μg/ml. The effect of cell density on efficiency was also examined, and slight but random variation was seen when concentration was raised from 1 × 10^6 to 3 × 10^6 cells per ml (data not shown). Increases in pulse duration or frequency were also found to affect transfection efficiency. When pulse duration was increased from 400 μs to 1 ms (at fixed frequency and field strength), efficiency decreased slightly. However, efficiency dropped significantly when pulse duration was further increased to 5 ms. It is interesting to note that a comparable percentage of viable cells obtained with different electrical

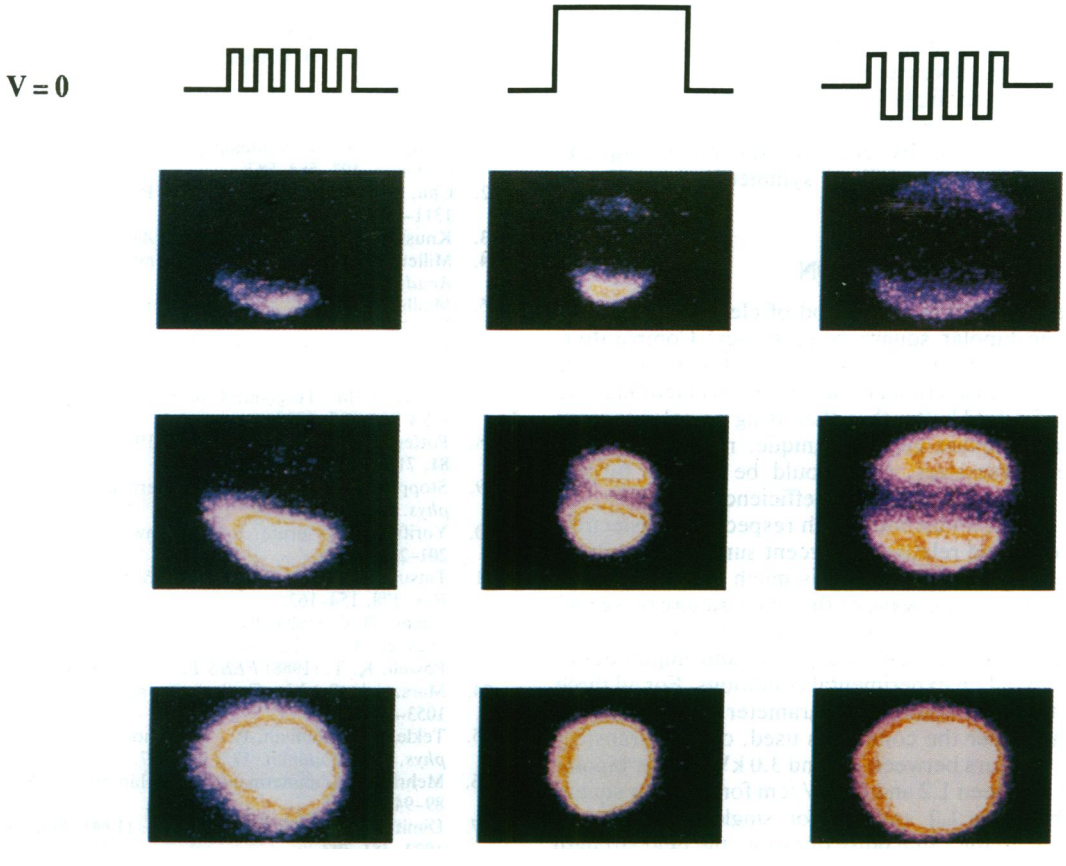

FIG. 5. Selected videoframes of electro-permeabilized cells subjected to the electric field of various wave forms. Ethidium bromide was used as a permeabilization-indicator probe; its fluorescence quantum yield is drastically enhanced when it binds to DNAs or RNAs. The positive electrode is at bottom, and the negative electrode is at top. In all frames, the fluorescence image before the electric pulse has been subtracted. Fluorescence is color coded in increasing intensity in the following order: blue, purple, gray, yellow, red, and white. Time increases from top to bottom. Pulse duration was 400 μs, and frequency was 250 kHz (when applied). (*Left*) Unipolar square wave; field strength: 1.10 kV/cm. Time series of frames was 0.528, 2.739, and 8.745 s. (*Middle*) Single square pulse; field strength: 4.95 kV/cm. Time series of frames was 0.297, 1.419, and 8.943 s. (*Right*) Bipolar square wave, field strength: 2.25 kV/cm (peak-to-peak). Time series of frames was 0.132, 0.891, and 3.267 s.

parameters for each wave form also differ notably in transfection efficiency (Table 1). Compare, for example, percent survival and efficiency at a pulse duration of 5 ms for each wave form with those observed for bipolar square wave at 3.0 kV/cm, and unipolar square wave and single square pulse at 2.2 kV/cm. These data indicate that not all surviving cells can be successfully transfected. When frequency was raised from 60 kHz to 1 MHz at a fixed field strength and pulse duration, a decrease in efficiency was seen despite the fact that percent survival increases under these conditions. This result is probably due to an insufficient time to reach the maximum induced membrane potential (24). In control experiments (Table 1), no resistant colonies were found.

Identification of Permeabilized Membrane Sites. Distinct qualitative differences between the bipolar and unipolar square wave or single square pulse in their ability to permeabilize the cell membrane in the region of electric field used for optimal transfection are shown in Fig. 5. The data clearly show that the cell membrane is permeabilized at either one hemisphere (that facing the positive electrode) or asymmetrically when the applied electric pulse is unipolar square wave or single square pulse. Further investigation indicated that the resting membrane potential may be responsible for the asymmetrical permeabilization observed (25). For earlier results on the issue of asymmetrical uptake see also refs. 26–28 and the references therein. In terms of permeabilizing the membrane, we found no qualitative difference between unipolar square wave and single square pulse. Fig. 5 *Left* shows the results obtained with a unipolar square wave. When the applied field is sufficiently enhanced, however, significant permeabilization is seen at both hemispheres facing the electrodes. Nevertheless, permeabilization remains asymmetric with the effect being more pronounced at that site facing the positive electrode. This asymmetric effect is demonstrated (using single square pulse) in Fig. 5 *Middle*. At these high fields the observed asymmetry may also derive from other factors (25). By contrast, the bipolar square wave-treated cell is permeabilized symmetrically, and the result is shown in Fig. 5 *Right*.

DISCUSSION

We report here an improved method of electro-permeabilization by using bipolar square wave pulses. Comparative study shows that under the optimal conditions for each method studied, transfection efficiency for this method is, at least, 1.7- and 5.5-fold better than that using unipolar square-wave and single square-pulse technique, respectively. By varying other parameters, one should be able to further optimize the observed transfection efficiency.

As seen in Fig. 4, a maximum with respect to transfection efficiency is obtained relative to percent survival. The maximum for the bipolar square wave is much greater than for either the unipolar square wave or the single square pulse and occurs at a higher percent survival; the peak is much sharper. We emphasize, however, that the locations and amplitudes of these peaks depend on experimental conditions. For all three pulse types, the main controlling parameter, as expected, is field strength. Under the conditions used, optimal transfection efficiency occurs between 1.6 and 3.0 kV/cm for bipolar square wave, between 1.2 and 2.2 kV/cm for unipolar square wave, and between 1.2 and 1.6 for single square pulse. Interestingly, with the same pulse duration, the field strength required for maximal transfection efficiency is $\approx\sqrt{2}$ higher for unipolar square wave than for a single square pulse, whereas the membrane potential is "on" twice as long in the latter case. Additionally, we note that the field amplitude (zero to peak) necessary for maximum transfection efficiency with the bipolar square pulse is $\approx 1/\sqrt{2}$ that required for the unipolar square pulse. This result may be related to the fact that both sides of the membrane are porated with bipolar pulses. The multiple permeabilized sites obtained with lower field strength, permits higher number of plasmid copies to be incorporated while survivability is still high. Therefore, the bipolar square-wave method is a better method for transfection relative to the other two wave forms used. We believe that this method should also provide similar advantages when applied to electrofusion experiments where contact between permeabilized areas is a critical factor for successful fusion and yield.

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