

## Surviving High-Intensity Field Pulses: Strategies for Improving Robustness and Performance of Electrotransfection and Electrofusion

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**Abstract.** Electrotransfection and electrofusion, both widely used in research and medical applications, still have to face a range of problems, including the existence of electroporation-resistant cell types, cell mortality and also great batch-to-batch variations of the transfection and fusion yields. In the present study, a systematic analysis of the parameters critical for the efficiency and robustness of electromanipulation protocols was performed on five mammalian cell types. Factors examined included the sugar composition of hypotonic pulse media (trehalose, sorbitol or inositol), the kinetics of cell volume changes prior to electropulsing, as well as the growth medium additives used for post-pulse cell cultivation. Whereas the disaccharide trehalose generally allowed regulatory volume decrease (RVD), the monomeric sugar alcohols sorbitol and inositol inhibited RVD or even induced secondary swelling. The different volume responses could be explained by the sugar selectivity of volume-sensitive channels (VSC) in the plasma membrane of all tested cell types. Based on the volumetric data, highest transfection and fusion yields were mostly achieved when the target cells were exposed to hypotonicity for about 2 min prior to electropulsing. Longer hypotonic treatment (10–20 min) decreased the yields of viable transfected and hybrid cells due to (1) the cell size reduction upon RVD (trehalose) or (2) the excessive losses of cytosolic electrolytes through VSC (inositol/sorbitol). Doping the plasma membrane with lipophilic anions prevented both cell shrinkage and ion losses (probably due to VSC inhibition), which in turn resulted in increased transfection and fusion efficiencies.

**Key words:** Cell volume regulation — Regulatory volume decrease — Osmotic stress — Trehalose — Inositol — Sorbitol — Volume-sensitive channels — Lipophilic anion — Electroporation — Electrorotation — Electrofusion

### Introduction

Electroinjection of membrane-impermeable molecules into living cells (so-called electroporation) and electrofusion of dielectrophoretically aligned cells are routinely used in many areas of biotechnology and bio-medicine for manipulation of the genome and cytosol of cells [18, 19, 33–35, 38]. Both electromanipulation techniques are based on the application of high-intensity electric field pulses of very short duration. A field pulse applied to a cell suspension induces a potential difference  $V_g$  across cell membranes, which in turn usually causes membrane breakdown when  $V_g$  is raised above  $\sim 1$  V at room temperature [39]. Membrane breakdown is reversible provided the pulse duration is not longer than several tens of microseconds. Under these conditions, the short-lived perturbations in the membrane structure (“electropores”), which provide transient access to the cytosol, reseal within a few minutes at increased temperatures (30° to 37° C). A wide range of molecules, including hormones, proteins, RNA, DNA, and even particles and organelles can be incorporated into living cells by electroporation without deterioration of cellular functions. In the case of two cells tightly attached to each other by dielectrophoresis, membrane breakdown in the contact zone can lead to cell fusion and subsequently to hybridization (for review, see [38]).

Best electroinjection and electrofusion yields (in terms of molecular uptake, transfection and hybridization rates) are frequently obtained if weakly conductive, hypotonic media are used during electropulsing [2, 6, 9, 23, 26, 27]. Such media usually contain a sugar derivative as the major solute. Among other sugars, monomeric carbohydrates, such as glucose, sorbitol, inositol or mannitol, as well as disaccharides, including sucrose or trehalose [3, 4, 10, 21, 28], are commonly used. Reducing both tonicity and conductivity of the pulse medium serves multiple purposes during electromanipulation [38]. Hypotonic cell swelling enhances membrane breakdown, simply because the induced membrane voltage  $V_g$  depends linearly on the cell radius. Decreasing the medium conductivity below that of the cytosol improves the molecular uptake by electroporated cells due to the transient electrodeformation force [20, 32]. In the case of electrofusion, low-conductive media are obligatory to align cells by positive dielectrophoresis and also to enhance the mutual attraction of fusion partners. Hypotonic pre-swelling of cells also facilitates electrofusion via the dissolution of the cytoskeleton, smoothing of cell membranes and increasing the mobility of membrane components [38].

Despite their wide applications, both electrotransfection and electrofusion still meet a series of challenges. These are the existence of electroporation-resistant cell types, mortality of electroporated cells, low transfection/fusion yields as well as their great batch-to-batch variations. In this study, we examined the parameters most crucial for the efficiency and robustness of electrotransfection and -fusion by systematically modifying standard protocols with respect to the (1) sugar composition of pulse media, (2) duration of hypotonic treatment prior to pulse application (including cell washing steps), (3) optimum additives to the pulse solutions as well as (4) nutrition medium used for post-pulse growth of transfected and hybrid cells.

Using time-resolved videomicroscopy, we found that hypotonic trehalose-substituted media generally allowed regulatory volume decrease (RVD) in all tested cell types. In contrast, sorbitol and inositol inhibited RVD or even induced secondary swelling. The difference between the disaccharide trehalose and the monomeric sugars could be explained by the size selectivity of volume-sensitive channels (VSC). Based on the volumetric data, the duration of hypotonic stress could be significantly optimized. A short-term hypotonic treatment prior to electropulsing (corresponding to the peak swelling at about 2 min upon hypotonic exposure) gave mostly highest transfection and fusion yields. Longer incubation times (e.g., 10–20 min) decreased the yields due to (1) the cell shrinkage via the RVD mechanism in trehalose and/or (2) the excessive leakage of cytosolic electrolytes through the permanently activated VSC in inositol- or

sorbitol-substituted media. The inhibition of VSC by doping the plasma membrane with a lipophilic tungsten carbonyl anion prevented both cell shrinkage and ion losses, which yielded an increase in transfection and fusion efficiencies. A further significant enhancement of fusion- and transfection efficiencies was achieved by sorting out the cytotoxic growth medium additives with lethal effects on cell hybrids and transfectants during post-pulse cultivation.

## Materials and Methods

### CHEMICALS, CELL CULTURE AND ELECTROMANIPULATION MEDIA

All sugars and sugar alcohols, including trehalose (*tre*), inositol (*ino*) and sorbitol (*sor*), as well as inorganic salts of highest purified grade, were purchased from Sigma, Fluka (both Deisenhofen, Germany), or Merck (Darmstadt, Germany). Bovine serum albumin and phytohemagglutinin (PHA-L) were supplied by Boehringer Ingelheim (Heidelberg, Germany) and Biochrom-Seromed (Berlin, Germany), respectively.

The tetraethylammonium salt of lipophilic tungsten carbonyl anion (hereafter denoted as LTC<sup>-</sup>), tetraethylammonium decacarbonyl- $\mu$ -dithioformiato-ditungstate  $\text{Et}_4\text{N}[\text{W}_2(\text{CO})_{10}(\mu\text{-S}_2\text{CH})]$ , was synthesized and purified by crystallization as described elsewhere [16]. LTC<sup>-</sup> was added to cell suspensions at a final concentration of 5–10  $\mu\text{M}$  from a 10 mM stock solution in ethanol. During microscopy, LTC<sup>-</sup>-treated cells were illuminated through a barrier filter ( $\lambda > 500 \text{ nm}$ ) in order to avoid the photochemical degradation of the highly photosensitive LTC<sup>-</sup>.

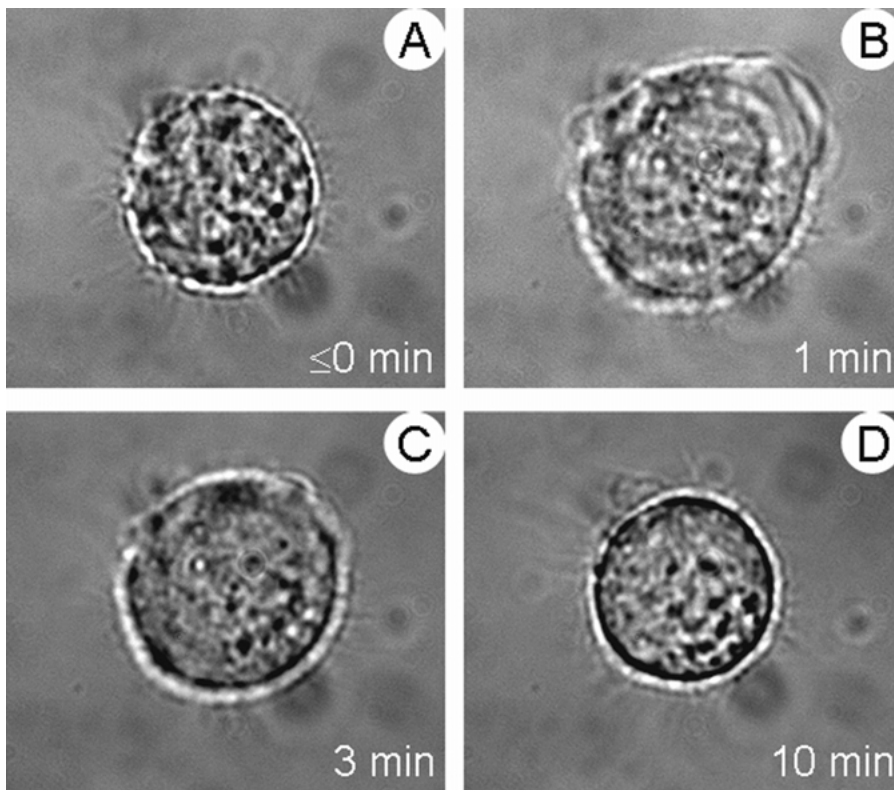
### CELLS

For electrotransfection, the human T-lymphocyte cell line Jurkat, the 293 human embryonic kidney cell line (HEK) and the murine fibroblast cell line L929 were cultured in RPMI 1640 complete growth medium (CGM), supplemented with 10% (v/v) fetal calf serum (FCS), at 37°C under 5% CO<sub>2</sub>. FCS was purchased either from Gibco (Grand Island, NY, USA) or from PAA (Linz, Austria). Cells were subcultured twice or thrice weekly. For cell passage, adherent L929 and HEK cells were detached by trypsinization (0.5 mg/ml trypsin and 0.2 mg/ml EDTA; PAA, Linz, Austria) from the culture flasks. For electrotransfection, the cells were detached by treatment with 0.5 mg/ml trypsin without EDTA in order to avoid the contamination of pulsing media with this chelating agent. The enzyme was removed by washing with CGM.

For electrofusion, human B-lymphocytes were isolated from the peripheral blood of healthy donors and activated with 2.4  $\mu\text{g/ml}$  PHA-L as described elsewhere [25, 38]. The activated cells were cultured at 37°C in a 5% CO<sub>2</sub>-enriched atmosphere. When cell density exceeded  $\sim 5 \times 10^6$  cells/ml, cultures were diluted to a density of  $1 \times 10^6$  cells/ml. The activated B-lymphocytes were fused with the human-mouse heteromyeloma cell line H73C11 (hereafter referred to as H7 cells, donated by St. Fount, Stanford, CA, USA; *see also* [37]), which was grown under standard conditions.

### CELL VOLUMETRY

Cell volumetry was performed in order to examine the swelling response of the different cell lines in strongly hypotonic electrotransfection and -fusion media. Cell volume changes were measured in a flow chamber designed for rapid exchange of media,



**Fig. 1.** Fast swelling and regulatory volume decrease (RVD) of an L929 cell in 100 mOsm medium containing trehalose as the major osmolyte. The microphotographs show the same cell before (*A*, isotonic CGM) and after acute hypotonic challenge (*B–D*) at the indicated time intervals. In isotonic CGM (*A*, time  $\leq 0$ ), the cell had a radius  $a$  of 8.7  $\mu\text{m}$ , corresponding to the original isotonic volume  $V_0 \approx 2.8$  pL. Upon medium replacement at zero time, the cell swelled to  $V_{\text{max}} \approx 5.0$  pL ( $r \approx 10.6$   $\mu\text{m}$ ) within 1 min (*B*) and then shrank gradually to its isotonic size within 3–10 min (*C–D*).

using the videomicroscopic setup described elsewhere [24]. Before measurements, an aliquot of cells suspended in isotonic CGM (270–300 mOsm) at a density of about  $10^5$  cells/ml was injected into the flow chamber and the cells were allowed to settle and to adhere to the glass coverslip (coated with poly-D-lysine) for 10–15 min. The cells were photographed 1 min before and at various time intervals (20–60 s) up to 25 min after medium exchange. The cross-section areas ( $A$ ) of 6–9 cells per microscopic field were determined. At each time interval, the volume ( $V$ ) of an individual cell (Fig. 1) was evaluated from its cross-section by assuming spherical geometry. The cell volume was normalized to the original isotonic volume ( $V_0$ ) as:  $v = V/V_0$ . The mean  $v$  values ( $\pm$  SE) for a given experiment were calculated from a sequence of about 80 images and plotted against time after the change from isotonic to hypotonic solution. Experiments for each sugar solution were performed on 2–3 separate cell passages.

### ELECTROROTATION

To determine the electrical cell properties, electrorotation (ROT) measurements were performed within the microstructured four-electrode chambers and experimental setup described in detail previously [31]. ROT spectra were monitored by decreasing the field frequency  $f$  in steps, from 150 MHz to  $\sim 1$  kHz. At each frequency, the rotation speed of lone cells located near the center of the chamber was determined using a stopwatch, and normalized to the field strength of 1 V<sub>pp</sub> per 100  $\mu\text{m}$ .

The ROT spectra of control untreated cells were approximated by the single-shell spherical model, which displays two Lorentzian peaks centered at the characteristic frequencies and  $f_{c1}$  and  $f_{c2}$ . As given in detail elsewhere [7, 15], the low-frequency anti-field peak ( $f_{c1}$ ) is mainly determined by the capacitive charging of the plasma membrane, whereas the co-field rotation ( $f_{c2}$ ) is primarily due to the cytosolic polarization.

Within a range of external conductivity ( $\sigma_e$ ), cells treated with lipophilic anions exhibit an additional anti-field maximum due to the relaxation of mobile charges within the plasma membrane [16]. The area-specific concentration  $N_i$  [pmol/cm<sup>2</sup>] and the translocation rate constant  $k_i$  [s<sup>-1</sup>] of the mobile charges (i.e., adsorbed lipophilic ions), were extracted by fitting the mobile-charge model [30] to the ROT spectra.

### ELECTROTRANSFECTION AND ELECTROINJECTION OF PROPIDIUM IODIDE (PI)

The electrotransfection protocols included the following steps: (1) washing of cells with an isotonic washing solution (IWS-P, see Table 1); (2) suspension of cells ( $10^6$  cells/ml) in a hypotonic pulsing solution (HPS, Table 1), containing 10  $\mu\text{g/ml}$  pEGFP-C1 (Clontech, Heidelberg, Germany) encoding for enhanced green fluorescence protein (GFP); (3) incubation of cells in HPS for 2 or 10 min; (4) application of a single exponentially decaying pulse to cell samples ( $\sim 800$   $\mu\text{l}$ ) at room temperature (RT  $\approx 22$ – $24^\circ\text{C}$ ) using the Multiporator (Eppendorf, Hamburg, Germany; www.eppendorf.com) and sterile cuvettes with plane aluminum electrodes spaced by 4 mm; (5) resealing, i.e., post-pulse incubation of cells in the pulse medium, for 10 min at RT; (6) the cells were then gently transferred to 5 ml prewarmed CGM without phenol red and cultured for 2 days in order to achieve the maximum GFP expression; (7) analysis by flow cytometry and electronic cell counting.

L929 cells were electrotransfected with a pulse of 3 kV/cm field strength and 100  $\mu\text{s}$  duration, in 100 mOsm pulse media (HPS, Table 1). Jurkat cells were treated with 1.2 kV/cm and 40  $\mu\text{s}$ , in 100 mOsm. HEK cells were electropulsed with 1.5 kV/cm and 70  $\mu\text{s}$ , in 150 mOsm pulse media (HPS, Table 1). These electrotransfection conditions, including the field pulse parameters and medium osmolality for each cell line, are based on the results of earlier studies [6, 21]. In a series of experiments, the pulse media contained

**Table 1.** Composition of washing and pulsing solutions used for electrotransfection

	Isotonic washing solution for poration IWS-P	Hypotonic pulsing solution HPS
Inositol or trehalose	240 mM	50 or 100 mM
KCl	25 mM	25 mM
KH <sub>2</sub> PO <sub>4</sub>	0.3 mM	0.3 mM
K <sub>2</sub> HPO <sub>4</sub>	0.85 mM	0.85 mM
pH value	7.4	7.4
Conductivity at 22–24°C	~3.5 mS/cm	~3.5 mS/cm
Osmolality	~290 mOsm	~100 or 150 mOsm

**Table 2.** Composition of media used in the improved electrofusion protocol

	Isotonic washing solution for fusion IWS-F	Hypotonic fusion solution HFS-75/HFS-100
Ca-acetate	0.1 mM	0.1 mM
Mg-acetate	0.5 mM	0.5 mM
Bovine serum albumin	1 mg/ml	1 mg/ml
Sorbitol	290 mM	75 or 100 mM
pH value	7.0	7.0
Conductivity at 22–24°C	~120 µS/cm	~120 µS/cm
Osmolality	~290 mOsm	~75 or 100 mOsm

25 µg/ml PI (Sigma), a cationic membrane-impermeable dye that reveals strong red fluorescence after binding to nucleic acids.

## FLOW CYTOMETRY AND DATA ANALYSIS

Measurements of GFP gene expression and PI uptake were performed in a flow cytometer Epics XL system (Beckman Coulter, Fullerton, CA, USA) equipped with a 15 mW 488 nm argon laser. Cellular green and red fluorescence (GF and RF) signals from samples containing about 5000 cells were measured using the band pass filters 525 and 675 nm, respectively (the bandwidth of the filters is 5 nm). The output GF or RF data were analyzed with the WinMDI software (TSRI, La Jolla, CA, USA) and presented as one-dimensional distributions of GFP or PI content. To discriminate between GFP-positive and GFP-negative cells a threshold of 0.5% was chosen by using the GF-histograms of untreated cells (for details *see* [20, 21]).

The number of viable cells was determined before and after pulse administration by electronic cell counting using a Coulter-like cell analyzer (Casy Cell Counter, Schärfe System, Reutlingen, Germany). Long-term cell viability was defined as the ratio of the viable cell count after 48 h cultivation and the initial cell number.

## ELECTROFUSION

The standard protocol for electrofusion of activated B-lymphocytes with H7 cells was elaborated in a series of earlier studies [25, 38]. According to this protocol, B-lymphocytes and H7 cells were mixed

in CGM in a ratio of 1:1, pelleted by centrifugation at 200 × *g* for 10 min and re-suspended in a hypotonic sugar-substituted fusion solution (HFS) of osmolality of 75 or 100 mOsm (*see* Table 2). The cells were then washed twice (centrifugation at 200 × *g* for 10 min and re-suspension) with HFS. 200 µl of the cell suspension in HFS were pipetted into a helical chamber consisting of a Perspex tube around which two platinum wires are wound 200 µm apart [38]. The final cell density was 3 × 10<sup>5</sup> cells/ml. According to this standard protocol the cells were subjected to hypotonic stress for at least 20 min during the two washing steps before the first fusion pulse is applied. In the improved protocols (*see* Results), the duration of hypotonic stress was significantly reduced by using isotonic washing solutions (IWS-F, Table 2).

The Eppendorf-Multiporator was used for electrofusion. The cells were first aligned dielectrophoretically by an alternating field of 5 V<sub>PP</sub> amplitude and 2 MHz frequency for 30 s. After that, the high frequency field was switched off and fusion was initiated by 3 rectangular DC pulses of 30 V amplitude and of 15 µs duration (*see* [25, 38] for more details). Then, a 2-MHz-field of 5 V<sub>PP</sub> amplitude was applied again for 30 s in order to keep the cells in position during the following fusion process. The helical chamber was then kept without disturbance for 10 min at RT. The chamber was then rinsed with 1 ml isotonic CGM without phenol red, and the cells were plated into 4 wells (filled with 1 ml isotonic CGM without phenol red) of a 24-well plate. After 24 h, the selection HAT-medium was added to the wells, in which only the hybridoma cells proliferated. Hybridoma colonies were counted 1–3 weeks after electrofusion.

## Results

### EFFECT OF GROWTH MEDIUM ADDITIVES ON ELECTROFUSION YIELD

It is well known that electroporated cells undergoing membrane resealing are very sensitive to some additives of pulse and nutrition media, such as the pH indicator phenol red, chelating agents (EDTA-like substances), etc., and also to traces of organic and inorganic contaminants (heavy-metal ions, peptides, etc.). Being completely harmless to unpulsed cells, these and other poorly defined contaminants may adversely affect electroporated cells thus decreasing the number of viable hybrid and transfected cells, and also leading to inconsistent batch-to-batch results.

Therefore, we first screened a number of growth medium additives for the highest survival rates of electrofused hybrid cells. We found that the hybridoma yield was subject to enormous variation (Table 3), even if lymphocytes from the same donor and identical electrofusion conditions were used. Comparison of the results obtained with eight different post-pulse growth media prepared from different RPMI and FCS batches (Table 3) revealed that the FCS batch had the dominant effect on the hybridoma yield. As seen from Table 3, the FCS lot number 3088682D from Gibco gave best results with two different RPMI batches tested here (Table 3, rows 1 and 3). Other FCS batches reduced the hybrid yield to about half (e.g., rows 2 and 4 in Table 3) or even inhibited completely the growth of hybridoma cells (e.g., row 8, Table 3), indicating

**Table 3.** Effect of the post-pulse growth medium on the clonogenic ability fusion products<sup>1</sup>

	RPMI 1640 Manufacturer / Cat. #	FCS Manufacturer /Lot #	Colony count <sup>2</sup>	Number of experiments <i>N</i>
1	Gibco / 32404	Gibco / 3088682D	219.2 ± 24.2	18
2	Gibco / 32404	Gibco / 16170-078	97.7 ± 27.3	6
3	PAA / E15-048	Gibco / 3088682D	197.3 ± 78.4	4
4	PAA / E15-048	Gibco / 16170-078	90–109	2
5	PAA / E15-048	Gibco / 40Q6214K	8.0 ± 2.6	6
6	PAA / E15-048	Sigma/24K3395	39–48	2
7	PAA / E15-048	PAA (Gold)	8.8 ± 5.3	6
8	X-Vivo Biowhittaker / BE04-744Q / 2MB0231 (containing an FCS substitute)		0	4

<sup>1</sup>Electrofusion was performed using the standard protocol in HFS-75.

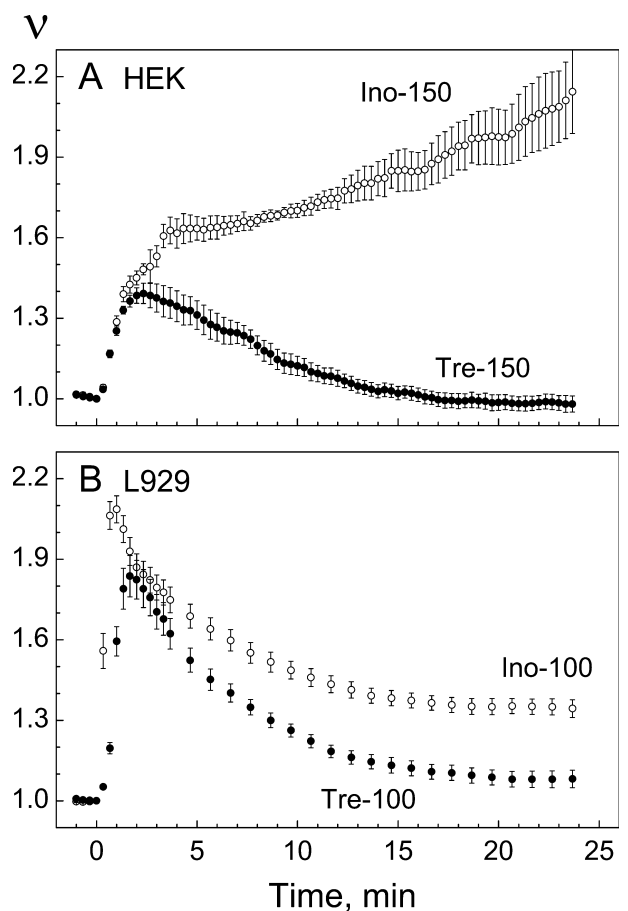
<sup>2</sup>The data are either the means ± SE or the ranges of two determinations.

that electropulsed cells were very sensitive to the FCS batch. Based on these data, the RPMI and FCS batches yielding the highest hybrid numbers (row 1 in Table 3) were used for post-pulse cultivation of electropulsed cells in all following electrofusion and electrotransfection experiments.

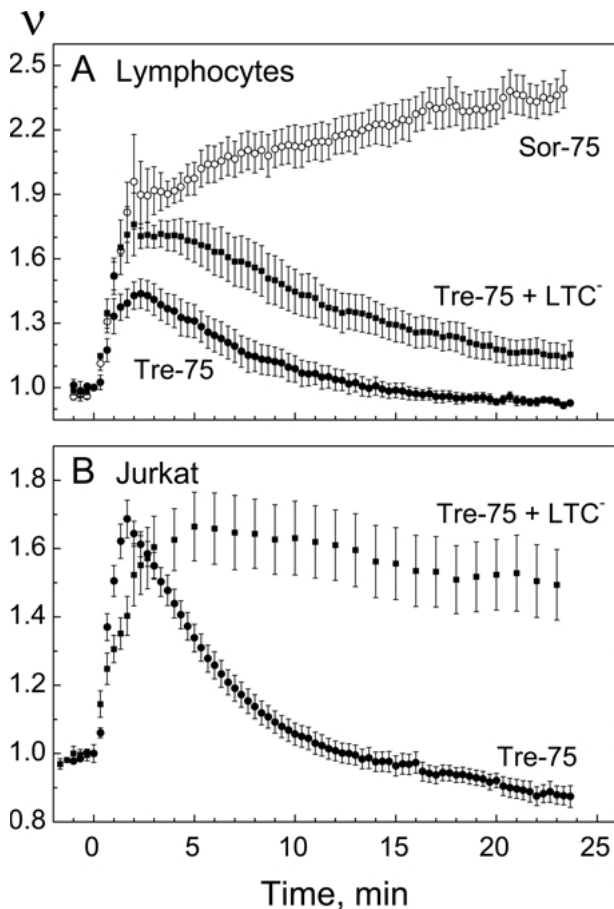
#### CELL VOLUME CHANGES IN HYPOTONIC MEDIA OF DIFFERENT COMPOSITION

The magnitude and time course of cell volume changes were studied by videomicroscopy after rapid transfer of various cell types from an isotonic saline (CGM, 270–300 mOsm) to a hypotonic electromanipulation solution. The hypotonic pulsing solutions (HPS) contained either inositol or trehalose as the major solute (Table 1). The osmolality of HPS was adjusted to 100 mOsm for L929 cells, and to 150 mOsm for HEK cell lines (according to standard electrotransfection protocols, *see* www.eppendorf.com). The hypotonic fusion solution (HFS, 75 mOsm) contained mainly sorbitol (Table 2). For comparison, 75 mOsm trehalose-substituted medium was also used in volumetric experiments. The volumetric response of Jurkat cells to hypotonic solutions of various sugars, including inositol and trehalose, has also been studied elsewhere [24].

In the presence of trehalose, the different cell lines studied here exhibited qualitatively similar transient volumetric responses to hypotonicity, with respect to both initial and secondary volume changes (Figs. 2 and 3, *filled circles*). Upon a sudden medium exchange, the cells swelled rapidly within the first 1–2 min from their original isotonic volume  $V_0$  to the maximum volume  $V_{max}$  due to the fast water uptake driven by the imposed osmotic gradient. After the initial swelling, the cells underwent regulatory volume decrease (RVD). Despite persisting hypotonicity, the cells shrank gradually during RVD and recovered their original isotonic volume ( $V_0$ ) within ~10–15 min



**Fig. 2.** Time courses of the relative volume ( $v = V/V_0$ ) of HEK (A) and L929 cells (B) in hypotonic electroperoration media of indicated osmolalities (150 or 100 mOsm). The media contained either inositol or trehalose as the major solute. In these experiments, the cell samples were originally (time < 0) exposed to isotonic saline medium (CGM). At zero time, CGM was replaced by a hypotonic sugar solution. After the fast initial swelling, both cell types exhibited complete RVD in hypotonic trehalose medium (*filled circles*). In contrast, secondary swelling on HEK cells or partial RVD in L929 cells occurred in inositol-substituted media (*empty circles*).



**Fig. 3.** Time courses of the relative volume of activated B-lymphocytes (*A*) and Jurkat cells (*B*) in strongly hypotonic fusion media (75 mOsm), containing either sorbitol or trehalose as the major solute. In hypotonic trehalose medium (*filled circles*), both cell types accomplished RVD, after the fast initial swelling. In contrast, hypotonic sorbitol abolished RVD in Jurkat cells (*data not shown*) or even induced secondary swelling in B-lymphocytes (*A*, *empty circles*).  $\text{LTC}^-$  (*filled squares*) inhibited RVD partially in B-lymphocytes or completely in Jurkat cells.

after hypotonic shock. Note that trehalose allowed RVD within a wide osmolality range (75–150 mOsm), in all mammalian cell lines used here.

In contrast to the disaccharide trehalose, both monomeric sugar alcohols inositol and sorbitol influenced the secondary volume changes and particularly RVD in a complex, cell type- and osmolality-dependent manner (Figs. 2 and 3, *empty circles*). Thus, the 150 mOsm inositol-substituted HPS abolished RVD in HEK cells (Fig. 2*A*, *empty circles*), whereas the 100 mOsm inositol-substituted HPS allowed partial RVD in L929 cells (Fig. 2*B*, *empty circles*) and also in Jurkat cells [24]. Moreover, the initial swelling of HEK cells was followed by a slower, but sustained secondary swelling in 150 mOsm inositol medium (Fig. 2*A*, *empty circles*). Similarly, 75 mOsm sorbitol (HFS) caused a

distinctly biphasic swelling response in activated B-lymphocytes (Fig. 3*A*, *empty circles*). The volume of H7 cells exposed to 75 mOsm sorbitol remained nearly unchanged after the fast initial swelling (*data not shown*).

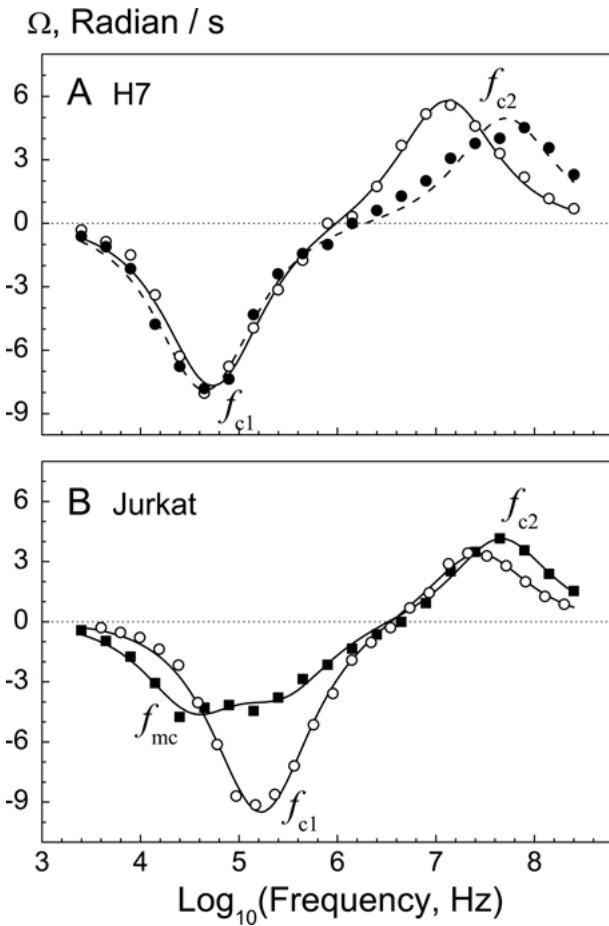
Micromolar concentrations of  $\text{LTC}^-$  exerted a strong influence on the secondary volume regulation in mammalian cells exposed to hypotonic trehalose-substituted medium. Thus, treatment of B-lymphocytes with 10  $\mu\text{M}$   $\text{LTC}^-$  led to a partial inhibition of RVD in trehalose-substituted medium (Fig. 3*A*, *filled squares*), whereas 5  $\mu\text{M}$   $\text{LTC}^-$  abolished RVD in Jurkat cells (Fig. 3*B*, *filled squares*). Measurements on other cell lines showed that, irrespectively of the sugar used,  $\text{LTC}^-$  and other lipophilic anions (*data not shown*) generally increased and stabilized the cell volume as compared to untreated control cells.

### ELECTROROTATION

To account for the possible changes in the electrical cell properties caused by hypotonic stress and  $\text{LTC}^-$ , electrorotation (ROT) measurements were additionally performed. The ROT technique allows the non-invasive determination of the electrical cell parameters, including the cell membrane capacitance  $C_m$  and conductance  $G_m$ , as well as the cytosolic conductivity  $\sigma_i$  and permittivity  $\epsilon_i$ . The ROT technique, however, does not offer sufficient time-resolution for continuous monitoring of cells undergoing fast volume changes induced by hypotonicity. Therefore, we analyzed the electrical cell properties after a prolonged hypotonic stress.

Prior to ROT measurements illustrated by Fig. 4*A*, H7 cells were incubated for 15–20 min in either hypotonic or isotonic medium (corresponding to HFS-75/100 or IWS-F, respectively, *see* Table 2). Compared to isotonic conditions (Fig. 4*A*, *filled circles*), incubation in strongly hypotonic medium resulted in a considerable shift of the co-field cytosolic peak (indicated by  $f_{c2}$ ) to lower frequencies, from  $\sim 50$  to 13 MHz. The position of the anti-field plasma membrane peaks ( $f_{e1}$ ) remained nearly unchanged. As discussed below, the strong low-frequency shift of the  $f_{c2}$  peak (Fig. 4*A*) implies that hypotonic treatment led to a significant reduction of  $\sigma_i$ .

The electrical parameters obtained by fitting the ROT spectra of H7 and Jurkat cells to the single-shell model (*curves* in Fig. 4) are summarized in Table 4. For both cell lines, prolonged incubation in hypotonic sorbitol-substituted medium resulted in a 3 to 4-fold reduction of the cytosolic conductivity  $\sigma_i$  (e.g., from  $\sim 11$  to 2.5 mS/cm for Jurkat cells), as well as in a marked decrease of the area-specific membrane capacitance  $C_m$  (from 1.3 to 0.7  $\mu\text{F}/\text{cm}^2$  for Jurkat cells). In hypotonic trehalose media, in which RVD occurred in all cell types (Figs. 2 and 3),  $\sigma_i$  also decreased (e.g., from 8.5 to 4.1 mS/cm for Jurkat cells,



**Fig. 4.** Typical rotation spectra of H7 and Jurkat cells (*A* and *B*, respectively). (*A*) H7 cells were incubated for 15–20 min in isotonic or hypotonic sorbitol media (*filled* and *empty symbols*, respectively), of the same conductivity of 120  $\mu\text{S}/\text{cm}$ . Compared to isotonic conditions, hypotonic stress led to a marked shift of the cytosolic peak  $f_{c2}$  to lower frequency indicating a significant reduction of cytosolic conductivity  $\sigma_i$ . The plasma membrane peak  $f_{c1}$  remained nearly unchanged. (*B*) Compared to untreated control (*open circles*), 10  $\mu\text{M}$   $\text{LTC}^-$  caused an additional anti-field peak ( $f_{mc}$ ), and also moved the  $f_{c2}$  to higher frequency. The curves are best least-square fits of the single-shell or mobile charge models to the data. For curve fitting, the membrane conductance  $G_m = 10 \text{ mS}/\text{cm}^2$  was assumed, as determined in preliminary experiments (*data not shown*) by the contra-rotating field technique [16, 31]. The electrical parameters, including  $C_m$ ,  $\sigma_i$ , and  $\epsilon_i$ , derived by curve fitting are summarized in Table 4. The mobile charge parameters  $N_t$  and  $k_i$  were:  $0.63 \pm 0.06 \text{ pmol}/\text{cm}^2$  and  $(3.7 \pm 0.5) \times 10^{-5} \text{ s}^{-1}$ , respectively.

Table 4), but to a lesser extent than in hypotonic sorbitol.

As illustrated in Fig. 4*B* (*filled squares*), treatment of Jurkat cells with 10  $\mu\text{M}$   $\text{LTC}^-$  led to drastic alterations in the ROT spectra. In contrast to untreated control cells (Fig. 4*B*, *empty circles*), whose ROT spectra exhibited a single anti-field peak ( $f_{c1} \approx 160 \text{ kHz}$ ), the spectra of  $\text{LTC}^-$ -treated cells displayed an additional low-frequency anti-field peak centered

at about 25 kHz. This peak (denoted by  $f_{mc}$ ) arose due to the relaxation of the adsorbed lipophilic anions or mobile charges within the membrane. Fitting the mobile charge model to the ROT spectra of several cells in 100 mOsm sorbitol yielded the mean values ( $\pm \text{SE}$ ) of the surface concentration of the adsorbed  $\text{LTC}^- N_t = 0.63 \pm 0.06 \text{ pmol}/\text{cm}^2$  and of its translocation  $k_i = (3.7 \pm 0.5) \times 10^{-5} \text{ s}^{-1}$ . Similar  $N_t$  and  $k_i$  values,  $0.66 \pm 0.06 \text{ pmol}/\text{cm}^2$  and  $(2.4 \pm 0.2) \times 10^{-5} \text{ s}^{-1}$ , respectively, were obtained in 100 mOsm trehalose.

In addition to its strong effect on the anti-field part of the ROT spectrum,  $\text{LTC}^-$  also shifted the cytosolic  $f_{c2}$  peak to higher frequency (Fig. 4*B*, *filled squares*), indicating an increased cytosolic conductivity  $\sigma_i$ , as compared to untreated control (Fig. 4*B*, *empty circles*). Despite significant swelling in 100 mOsm sorbitol ( $v \approx 1.8$ ), treatment of cells with  $\text{LTC}^-$  led to a two-fold increase of  $\sigma_i$  from 2.5 to 5.3 mS/cm in Jurkat cells (Table 4). In 100 mOsm trehalose,  $\text{LTC}^-$  abolished RVD ( $v \approx 1.5$ , Table 4) and also increased  $\sigma_i$  from 4.1 to 5.7 mS/cm, which was apparently due to the ability of  $\text{LTC}^-$  to prevent excessive leakage of cytosolic electrolytes.

#### INFLUENCE OF HYPOTONIC STRESS DURATION ON ELECTROMANIPULATION

##### *Electrotransfection*

The optimum field conditions, medium tonicity and plasmid concentration for electrotransfection of the various mammalian cell lines, using standard hypotonic pulse media, were reported elsewhere [6, 21, 36]. Based on these standard protocols, we assessed in the present study the effect of the duration of hypotonic stress on the intracellular delivery and expression of the plasmid pEGFP in three different cell lines, including HEK, L929 and Jurkat cells. For each cell line, the hypotonic pulse media were tested that contained either inositol or trehalose as the major solute. The cells were exposed to hypotonic stress in the presence of either sugar for 2 or 10 min prior to electropulsing. The selected incubation times corresponded, respectively, to the peak cell volumes  $v_{\text{max}}$  (2 min) achieved after the fast initial swelling and to the completion of RVD (10 min) in trehalose-substituted media (Fig. 2).

Table 5 summarizes the results of electrotransfection experiments on HEK and L929 cells. The means  $\pm \text{SE}$  for the transfection yields, GFP gene expression, and cell proliferation from several measurements 48 h after electropulsing are given in the table, from which the following general trends are apparent.

In inositol medium, extending pre-pulse incubation of HEK cells from 2 to 10 min resulted in a somewhat higher transfection yield (*TY*) and also in a

**Table 4.** Electrical properties of H7 and Jurkat cells extracted from ROT spectra measured in iso- and hypotonic media of different sugar compositions<sup>a</sup>

Sugar and osmolality mOsm	LTC <sup>-</sup> $\mu$ M	Relative cell volume $v = V/V_0^b$	$\sigma_i$ mS/cm	$\varepsilon_i/\varepsilon_0$	$C_m$ $\mu$ F/cm <sup>2</sup>	Number of measurements
<b>H7 cells</b>						
Sorbitol 270		1	5.5 $\pm$ 0.2	88 $\pm$ 4	1.0 $\pm$ 0.1	9
Sorbitol 75		1.3 $\pm$ 0.1	2.2 $\pm$ 0.2	58 $\pm$ 4	0.5 $\pm$ 0.1	8
<b>Jurkat cells</b>						
Sorbitol 300		1	11.0 $\pm$ 10	150 $\pm$ 10	1.3 $\pm$ 0.1	12
Sorbitol 100		1.8 $\pm$ 0.1	2.5 $\pm$ 0.1	140 $\pm$ 5	0.7 $\pm$ 0.1	8
Sorbitol 100	10	1.8 $\pm$ 0.1	5.3 $\pm$ 0.3	88 $\pm$ 5	0.7 $\pm$ 0.1	7
Trehalose 300		1	8.5 $\pm$ 0.7	110 $\pm$ 10	1.3 $\pm$ 0.1	9
Trehalose 100		1	4.1 $\pm$ 0.2	120 $\pm$ 20	1.1 $\pm$ 0.1	8
Trehalose 100	10	1.5 $\pm$ 0.1	5.7 $\pm$ 0.2	110 $\pm$ 12	0.7 $\pm$ 0.1	12

<sup>a</sup>The mean  $C_m$ ,  $\sigma_i$  and  $\varepsilon_i$  values ( $\pm$  SE) were derived by fitting the single-shell model to the ROT spectra of cells such as shown in Fig. 4 (not treated with LTC<sup>-</sup>). For further details *see* legend to Fig. 4.

<sup>b</sup>The  $v$  values were obtained from the volumetric measurements such as shown in Figs. 2 and 3. The data represent the relative cell volume measured 20–25 min after hypotonic shock.

**Table 5.** Effects of duration of hypotonic stress and LTC<sup>-</sup> on transfection yield (TY), GFP gene expression (GE), and proliferation factor (PF) of HEK and L929 cells

Sugar and osmolality mOsm	Hypotonic stress duration $T$ , min <sup>a</sup>	LTC <sup>-</sup> $\mu$ M	Relative cell volume $v(T)^a$	TY <sup>b</sup> %	GE <sup>b</sup> a.u.	PF <sup>c</sup> %	TE <sup>d</sup> %
<b>HEK cells</b>							
Inositol 150	2		1.5 $\pm$ 0.1	44 $\pm$ 4	80 $\pm$ 7	47 $\pm$ 8	20.5
Inositol 150	10		1.7 $\pm$ 0.1	58 $\pm$ 9	158 $\pm$ 34	33 $\pm$ 4	19.1
Inositol 150	10	10	1.9 $\pm$ 0.2	44 $\pm$ 1	77 $\pm$ 8	47 $\pm$ 3	20.7
Trehalose 150	2		1.4 $\pm$ 0.1	35 $\pm$ 3	83 $\pm$ 5	68 $\pm$ 14	23.9
Trehalose 150	10		1.1 $\pm$ 0.1	20 $\pm$ 5	58 $\pm$ 7	56 $\pm$ 6	11.4
Trehalose 150	10	10	1.4 $\pm$ 0.1	38 $\pm$ 3	100 $\pm$ 12	59 $\pm$ 8	22.4
<b>L929 cells</b>							
Inositol 100	2		1.9 $\pm$ 0.1	85 $\pm$ 1	182 $\pm$ 10	16 $\pm$ 2	13.7
Inositol 100	10		1.5 $\pm$ 0.1	75 $\pm$ 4	104 $\pm$ 19	12 $\pm$ 1	9.1
Inositol 100	10	10	1.8 $\pm$ 0.1	87 $\pm$ 1	355 $\pm$ 22	16 $\pm$ 2	14.1
Trehalose 100	2		1.8 $\pm$ 0.1	71 $\pm$ 1	177 $\pm$ 21	31 $\pm$ 4	21.9
Trehalose 100	10		1.3 $\pm$ 0.1	60 $\pm$ 5	133 $\pm$ 28	28 $\pm$ 2	16.8
Trehalose 100	10	10	1.5 $\pm$ 0.2	69 $\pm$ 2	188 $\pm$ 10	45 $\pm$ 9	31.0

<sup>a</sup> $T$  is the duration of hypotonic treatment prior to electropulsing;  $v(T)$  is the relative cell volume at time  $T$  of pulse application after hypotonic shock.

<sup>b</sup> $TY$  and  $GE$  values represent the percentage and geometric mean fluorescence intensity of GFP-positive cells, respectively. The geometric mean  $GE$  values of non-transfected cells (i.e., the autofluorescence) were  $3 \pm 0.3$  a.u. The data represent the mean  $\pm$  SE of 3–9 experiments.

<sup>c</sup>PF is the number of viable cells 48 h after electrotransfection, given in percentage referred to the initial number of cells.

<sup>d</sup>Transfection efficiency  $TE$  is defined as  $TY \times PF$ , given in per cent.  $TE$  is proportional to the number of viable cells expressing the reporter gene.

nearly twofold increase of the GFP content given in arbitrary gene expression units ( $GE$ ). These effects, however, were achieved at the expense of cell viability, as seen from the reduced value of proliferation factor ( $PF$ ). In addition to the three measured parameters  $TY$ ,  $GE$  and  $PF$ , we also introduced the transfection efficiency index ( $TE$ ) defined as  $TY \times PF$  (in percentages). For HEK cells electroporated in hypotonic inositol, the  $TE$  index and thus the number of viable cells expressing GFP gene remained nearly unchanged when the pre-pulse incubation was extended (Table 5).

In the case of L929 cells, increasing the duration of hypotonic treatment from 2 to 10 min in inositol medium led to a reduction of both  $TY$  and  $GE$ . Prolonged hypotonic stress in inositol medium further suppressed proliferation of L929 cells as seen from the PF decrease from 16 to 12%. The reduction of PF was the main reason for the significant decrease in  $TE$  from 13.7 to 9.1%. In the presence of inositol, the effects of hypotonic stress duration on the transfection and proliferation of Jurkat cells (*data not shown*) were qualitatively similar to those obtained with L929 fibroblasts. It is noteworthy that in sharp



contrast to HEK cells, which exhibited secondary swelling, both Jurkat [24] and L929 cells (Fig. 2B) experienced partial RVD in hypotonic inositol medium. These findings are reflected in the different trends for  $\nu$  of HEK- and L929 cells with increasing the time of hypotonic stress (*see*  $\nu$  data in Table 5).

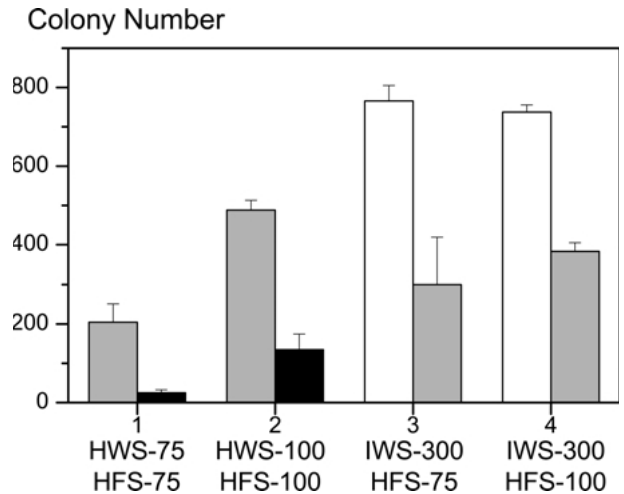
In the case of the short-duration hypotonic treatment (2 min), all transfection and proliferation parameters, including *TY*, *GE*, *PF* and *TE*, obtained for HEK cells in trehalose-substituted medium were very similar to the corresponding data obtained with this cell line in inositol medium. In contrast to inositol solutions, increasing duration of hypotonic stress in the presence of trehalose reduced the transfection efficiency to half (from 23.9 to 11.4%) by diminishing both *TY* and *PF* in HEK cells. Moreover, the excessive 10 min-exposure to hypotonic trehalose yielded the lowest gene expression ( $GE = 58$  a.u.) observed in this study.

Unlike HEK cells, L929 fibroblasts exhibited an improved transfection efficiency after the short hypotonic treatment with trehalose substituted medium. Although *TY* and *GE* were only slightly affected, trehalose substitution yielded a nearly twofold increase of *PF* ( $\approx 31\%$ ) and also a marked improvement of *TE* ( $\approx 22\%$ ) as compared to inositol medium ( $PF \approx 16\%$  and  $TE \approx 14\%$ ). The ability of trehalose to improve electrotransfection has been reported earlier [21]. As with HEK cells, increasing duration of hypotonic treatment in trehalose medium from 2 to 10 min resulted in a significant reduction of *TE* in L929 cells from about 22 to 17%.

### Electrofusion

Based on the standard electrofusion protocol for activated B-lymphocytes and H7 heteromyeloma cells (*see* Materials and Methods), the following set of experiments was performed in order to analyze the impact of hypotonic stress duration on electrofusion efficiency. Most conditions, including both AC and DC field strengths, pulse numbers, cell densities, fusion solution compositions and conductivities (Table 2), growth media (Table 3, line 1), etc., were kept constant in the course of these experiments. The only condition that was varied included the time interval between hypotonic shock and application of the first fusion pulse. The reduction of hypotonic stress duration was achieved by using isotonic sorbitol-substituted media (IWS-F, *see* Table 2) for cell washing prior to electrofusion.

Compared to standard protocols, in which cells are exposed to strongly hypotonic solutions (75 or 100 mOsm) for at least 20 min (Fig. 5, *gray bars* 1 and 2), the reduction of hypotonic stress to about 2 min yielded significantly larger numbers of hybridoma colonies for both tested osmolalities (Fig. 5, *white bars* 3 and 4). In the case of 75 mOsm fusion solutions (HFS-



**Fig. 5.** Effect of the duration of hypotonic stress prior to electrofusion on the yield of viable hybrids between human B-lymphocytes with the heteromyeloma cell line H7. In all experiments (1–4), the same AC alignment and DC pulse field conditions were used (*see* Materials and Methods). In experiments 1 and 2, hypotonic solutions of 75 and 100 mOsm were used for both washing and fusion. In these experiments, hypotonic washing solutions (HWS-75 and HWS-100) were identical to the corresponding fusion solutions (HFS-75 and HFS-100). In experiments 1 and 2, the total durations of hypotonic stress prior to electropulsing were about 20 and 40 min, indicated by gray and black bars, respectively. In experiments 3 and 4, the duration of hypotonic stress was reduced to  $\sim 2$  min by using isotonic washing solutions (IWS-F, *white bars*) instead of hypotonic ones. In experiments 3 and 4, additional 20 min incubation of cells in hypotonic media (HFS-75 and HFS-100, respectively) prior to fusion pulse application is indicated by gray bars.

75), the use of the isotonic washing solution (IWS-F) increased the colony number from about 200 to 800 (Fig. 5, *gray bar* 1 and *white bar* 3), thus improving nearly 4-fold the fusion efficiency. Similarly, isotonic washing also enhanced electrofusion in 100 mOsm media (HFS-100, Fig. 5, *gray bar* 2 and *white bar* 4), but to a somewhat lesser degree than in HFS-75.

Figure 5 also illustrates that increasing the duration of hypotonic stress prior to electropulsing led to a considerable reduction of fusion efficiency, in all tested protocols. Thus, in the standard protocol using 75 mOsm (bars 1, HWS/HFS-75), increasing duration of hypotonic treatment from  $\sim 20$  to  $\sim 40$  min prior to pulse application reduced 8-fold the number of hybridoma colonies, from  $\sim 200$  to 25 colonies (*gray and black bars* 1, Fig. 5). When isotonic washing media (IWS-F) were used, the colony numbers were also considerably reduced after increasing hypotonic stress duration from 2 to 20 min in HFS-75 and HFS-100 media (*gray bars* 3 and 4, Fig. 5). The results presented in Fig. 5 clearly show that long-term exposure of cells to hypotonic solutions should be avoided during the electrofusion procedure.

EFFECT OF LTC<sup>-</sup> ON ELECTROPORATION

As already mentioned, the cell size is one of the key parameters determining the degree of membrane permeabilization in electroporated cells. Based on the observation that LTC<sup>-</sup> stabilized the cell size by inhibiting RVD in hypotonic media (Fig. 3, Table 4), we further explored the influence of this lipophilic anion on the transfection parameters after prolonged hypotonic stress. Prior to electropulsing, the cells were incubated for about 10 min in hypotonic, inositol- or trehalose-substituted medium, which was additionally supplemented with 10  $\mu$ M LTC<sup>-</sup>.

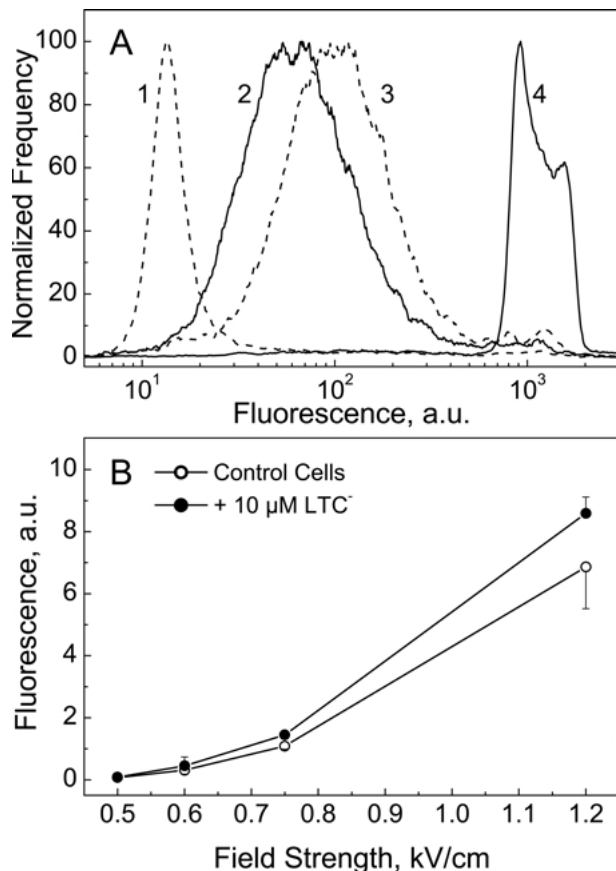
Judging by the transfection parameters given in Table 5, inclusion of 10  $\mu$ M LTC<sup>-</sup> in the trehalose-substituted pulse medium consistently increased gene expression (GE) as well as transfection efficiency (TE) after long-term hypotonic stress in both HEK and L929 cell lines. In the case of L929 cells, the rise in TE from 16.8 to 31% was mainly due to the improved proliferation ability (PF), whereas TY was only slightly increased (from 60 to 69%). In HEK cells, the lipophilic anion gave a nearly two-fold increase in TE from 11.4 to 22.4%, as a result of a marked TY improvement (from 20 to 38%) in trehalose-substituted medium.

Supplementation of inositol medium with LTC<sup>-</sup> also significantly improved the TE value of electrotransfected L929 cells from 9.1 to 14.1%, which was comparable to the TE value of 13.7% obtained after the optimum short-term hypotonic stress. Surprisingly, LTC<sup>-</sup>-treated L929 cells exhibited a more than 3-fold higher GE value (355 a.u.) as compared to control L929 fibroblasts (GE  $\approx$  104 a.u.).

In addition to GFP expression, we also used propidium iodide (PI) as the fluorescent reporter molecule to assess the degree of membrane electroporation. In these experiments, the pulse medium (HPS, Table 1) was supplemented with 25  $\mu$ g/ml PI. The electrically induced PI uptake was analyzed by flow cytometry 10 min after pulse application.

Typical PI content distributions (RF histograms) of unpulsed control, electropulsed and saponin-lyzed Jurkat cells suspended in hypotonic pulse media are shown in Fig. 6A (curves 1, 2 and 4, respectively). The histogram of control unpulsed cells in the presence of PI (Fig. 6A, curve 1) exhibited a very weak fluorescence peak centered at  $\sim$ 10 arbitrary units (a.u.). Treatment of cells with the detergent saponin resulted in 100% permeabilization and gave rise to a very high fluorescence intensity ranging from about 700 to 2000 a.u. (Fig. 6A, curve 4), which corresponded to near saturation of the intracellular binding sites for PI [20].

The RF histogram of electropulsed cell samples (Fig. 6A, curve 2) differed markedly from those observed in both control and saponin-lyzed cell cul-



**Fig. 6.** (A) Typical histograms of PI-DNA fluorescence of unpulsed control (1), electropulsed (2 and 3) and saponin-lyzed Jurkat cells (4) in HPS (see Table 1) containing 25  $\mu$ g/ml PI. Histograms 2 and 3 were measured 10 min after application of a field pulse of 1.2 kV/cm strength and 40  $\mu$ s duration generated by means of the Eppendorf-Multiporator. Curve 3 represents a cell sample treated with 10  $\mu$ M LTC<sup>-</sup>. (B) Dependence of the electrically induced PI uptake on the applied field strength  $E_0$  at a pulse duration of 40  $\mu$ s. The data points are the mean PI uptake values evaluated from 3 independent flow cytometric determinations, such as shown in A. Within the studied range of the field strength, the PI uptake in cells treated with 10  $\mu$ M LTC<sup>-</sup> (filled circles) was larger than in control (empty circles).

tures. Upon exposure of an electric field pulse (1.2 kV/cm, 40  $\mu$ s) in the presence of PI, two subpopulations of cells emerged in the histogram. About 5% of cells were irreversibly permeabilized, as suggested by their very bright RF similar to that of the saponin-lyzed samples. The second subpopulation included cells with much lower PI staining (i.e., far from equilibrium with the medium), whose peak signal was centered at about 60 a.u. These weakly fluorescent viable cells were transiently permeabilized and then recovered their original membrane impermeability (i.e., resealed) within few minutes after electropulsing [28].

The field-strength dependence of the mean PI fluorescence in electroporated cells is illustrated in Fig. 6B. The electrically induced PI uptake detectable

by flow cytometry was observed at field strengths  $E_0 \geq 0.6$  kV/cm. Above this threshold value, the PI fluorescence in viable cells grew gradually with increasing field strength. Within the field-strength range studied here (0.6–1.2 kV/cm), electroporation of cells treated with  $10 \mu\text{M}$  LTC<sup>-</sup> resulted generally in a larger PI uptake (Fig. 6B, *filled circles*), as compared to untreated control (Fig. 6B, *empty circles*). This result compares favorably to the increased efficiency of GFP transfection in LTC<sup>-</sup> treated cells (Table 5).

## Discussion

In the present study, a significant improvement in performance and robustness of electrotransfection and electrofusion of mammalian cells was achieved by (1) accounting for the cell-type specific volume regulation in hypotonic media of different sugar composition, (2) adjusting the duration of hypotonic treatment prior to pulse application, (3) stabilizing the cell volume via pulse medium supplementation with a lipophilic anion, (4) sorting out cytotoxic additives from nutrition media used for post-pulse growth of transfected and hybrid cells.

### EFFECTS OF VOLUME REGULATION ON ELECTROTRANSFECTION AND FUSION

#### *Solute Fluxes during RVD and Secondary Swelling*

Until now little attention has been paid to the complex volume response of mammalian cells to hypotonic electromanipulation media. We showed here that, after the fast initial swelling in such media, either secondary cell swelling or shrinkage occurred depending on the cell type and medium composition.

As illustrated in Figs. 2 and 3, the cells suddenly exposed to hypotonicity swelled rapidly due to the fast water influx from their original isotonic volume ( $v_0 = 1$ ) to the maximum volume  $v_{\text{max}}$ . The cells used here usually accomplished the “water phase” within 1.5–2 min after hypotonic shock. In general, the measured  $v_{\text{max}}$  data obtained here for different cell lines were much lower than those predicted for an ideal osmometer. Thus, the reduction of osmolality from 300 to 75 mOsm gave only a  $v_{\text{max}}$  value of 1.5–1.9 in activated B-lymphocytes (Fig. 3B), instead of the theoretically expected 4-fold volume increase (i.e., the reciprocal osmolality ratio).

Moreover, the volumetric data in Figs. 2 and 3 also revealed a dramatic difference between sorbitol and inositol on the one hand, and trehalose on the other in their effects on the secondary volume changes in the mammalian cell lines used here. Whereas the disaccharide trehalose generally allowed RVD in mammalian cells (*filled symbols* in Figs. 2 and 3), the

monomeric sugar alcohols inhibited RVD partially or completely, or even induced secondary swelling (*empty symbols* in Figs. 2 and 3). These findings confirm the results of an earlier study [24], in which the volumetric response of Jurkat cells to hypotonic media substituted with a variety of mono- and oligomeric sugars was examined.

Volume regulation in hypotonic media is a near-ubiquitous property of animal cells. Even under continuous hypotonic stress imposed by decreased external osmolality, most cells can re-adjust their normal isotonic volume after transient swelling by the RVD mechanism. During RVD, the initial cell swelling induces co-activation of volume-sensitive Cl<sup>-</sup> and K<sup>+</sup> channels, thus leading to the net efflux of KCl, osmotically inevitable water loss and to the restoration of normal cell volume [8, 17, 22]. As outlined elsewhere [24], the different effects of sugars on the secondary volume response of mammalian cells can be explained by the presence of volume-sensitive channels (VSC) conducting selectively small inorganic ions and monomeric sugars but being poorly permeable for oligosaccharides.

In the presence of an inward-directed sugar gradient (i.e., under the experimental conditions used here), the influx of monomeric sugar alcohols inhibited or abolished RVD by compensating the efflux of intracellular electrolytes from swollen cells. Therefore, enduring activation of volume-sensitive channels in hypotonic ion-deprived solutions of monomeric sugars (such as used for electrofusion, Table 2) can lead to a substantial loss of cytosolic ions. The expected depletion of the intracellular electrolyte was confirmed by the results of our electrorotation experiments (Fig. 4, Table 4), which revealed a marked reduction of the cytosolic conductivity  $\sigma_i$  in hypotonically treated cells, as compared to isotonic conditions.

Taking the volumetric and ROT data together, prolonged hypotonic exposure of cells prior to electropulsing can lead not only to marked transient alterations in cell size but also to a considerable reduction of the cytosolic conductivity. Both effects may influence via different mechanisms the response of target cells to field-pulse application, thus affecting the efficiency of electromanipulation protocols.

#### *The Impact of Transient Cell Size Changes*

The theory of electroporation of cell membranes is given in full details elsewhere [19, 33, 38]. In response to an applied DC electric field  $E_0$ , the voltage generated across the plasma membrane of a spherical cell grows exponentially with time:

$$V_{g(t)} = 1.5 a E_0 \cos \theta (1 - \exp(-t/\tau_m)) \quad (1)$$

where  $a$  is the cell radius,  $\theta$  is the polar angle measured with respect to field  $E_0$ . The relaxation time

constant  $\tau_m$  of the plasma membrane charging is given by:

$$\tau_m = aC_m \left( \frac{1}{\sigma_i} + \frac{1}{2\sigma_e} \right). \quad (2)$$

Using typical parameters for mammalian cells, i.e.,  $a = 10 \mu\text{m}$ ,  $C_m = 1 \mu\text{F}/\text{cm}^2$  and  $\sigma_i = 5 \text{ mS}/\text{cm}$  (Table 4), the  $\tau_m$  values of 4.37 and 0.34  $\mu\text{s}$  are obtained for cells suspended, respectively, in electrofusion (HFS,  $\sigma_e = 0.12 \text{ mS}/\text{cm}$ ) and transfection solutions (HPS, 3.5 mS/cm), by applying Eq. 2. Note that the estimated  $\tau_m$  values are much smaller than the corresponding pulse durations of 15  $\mu\text{s}$  and 40–100  $\mu\text{s}$  used here for electrofusion and electrotransfection, respectively. Therefore, Eq. 1 can be simplified to the well known steady-state expression:  $V_g = 1.5 aE_0 \cos \theta$ .

Consideration of Eq. 1 shows that the cell size is undoubtedly an important factor in determining what fraction of the cell surface will be electropermeabilized by a pulse of given field strength. Accordingly, the electrosensitivity of larger cells is usually higher than that of smaller ones, as supported by a number of studies on size heterogeneous cell populations [12].

In the present work, transient changes in cell size seemed to be the main reason for the effects of hypotonic stress duration on electrotransfection efficiency. In trehalose-substituted medium, electropulsing of large cells during the peak swelling stage  $v_{\text{max}}$  (corresponding to the 2 min-hypotonic stress) gave the highest transfection yields and gene expression in both HEK and L929 cell lines (Table 5). Electrotransfection of shrunken cells, i.e., during or after completing the RVD process (10 min in hypotonic trehalose medium), was less efficient with respect to both *TY* and *GE*. As expected from the size dependence of membrane breakdown (Eq. 1), electrotransfection benefited from the secondary swelling of HEK cells in inositol-substituted medium (Table 5, Fig. 2A, *empty symbols*), whereas partial RVD in L929 cells in this medium caused lower transfection and gene expression efficiencies (Fig. 2A and B, *empty symbols*).

### The Importance of Cytosolic Conductivity

Although cell-size changes during RVD can explain the observed variations of the transfection yields and gene expression, the size-dependence of electropermeabilization does not account for the results of electrofusion experiments (Fig. 5). Thus, despite secondary swelling of lymphocytes (Fig. 3B, *empty circles*), increasing the duration of hypotonic stress led to a marked reduction of hybridoma colonies (Fig. 5, *gray and black bars*), as compared to optimum conditions (Fig. 5, *white bars*).

As already mentioned, long-term incubation of cells in hypotonic fusion medium resulted in a substantial ion loss from the cells, thus leading to a strong decrease in the cytosolic conductivity  $\sigma_i$  (Table 4). The  $\sigma_i$  decrease can adversely affect the electrofusion efficiency by altering the polarization response of cells to the AC field imposed during dielectrophoretic alignment. In the 2 MHz field used here, the induced cell dipole  $\mu_C$  is given by the following equation [15, 20]:

$$\mu_C = 4\pi\epsilon_e\epsilon_0 a^3 E_0 \frac{\sigma_i - \sigma_e}{\sigma_i + 2\sigma_e}, \quad (3)$$

where  $\epsilon_e\epsilon_0$  and  $\sigma_e$  are the permittivity and conductivity of suspending medium. It is apparent from Eq. 3 that decreasing  $\sigma_i$  will reduce the induced dipole moment  $\mu_C$ , thus weakening not only the dielectrophoretic force but also the mutual attraction between adjacent cell dipoles. Since both electrokinetic interactions are essential for the creation of tight cell-to-cell contact, their reduction will certainly compromise the electrofusion efficiency.

### MULTIPLE EFFECTS OF LTC<sup>-</sup> ON ELECTROMANIPULATED CELLS

In addition to the well-known large organic ions dipicrylamine (DPA<sup>-</sup>), tetraphenylborate (TPB<sup>-</sup>), and related compounds, we have recently identified a number of anionic tungsten pentacarbonyl derivatives (including LTC<sup>-</sup> used here) as a new class of hydrophobic anions with high affinity to mammalian cell membranes [16, 31]. Unlike most ions and charged molecules, hydrophobic ions readily adsorb to lipid membranes and modify the intrinsic electrostatic potentials within the bilayer, including the surface  $\psi_S$  and dipole potentials  $\psi_D$  [5, 29], thus influencing various membrane transport systems [1, 11]. It can therefore be expected that doping of cell membranes with lipophilic ions can also affect the ion channels and carriers involved in cell volume regulation. In addition to the electrostatic effects on the bilayer, direct interactions of LTC<sup>-</sup> with ion transport systems of the cell membrane can not be excluded.

### Cell Volume Regulation

The above considerations prompted us to examine the effects of lipophilic anions on RVD in mammalian cells. As seen from Fig. 3, micromolar concentrations of LTC<sup>-</sup> inhibited partially RVD in B-lymphocytes exposed to hypotonic trehalose (Fig. 3A, *filled squares*). In Jurkat cells, LTC<sup>-</sup> caused complete inhibition of RVD (Fig. 3B, *filled squares*). Similar to LTC<sup>-</sup>, several other lipophilic anions (including DPA<sup>-</sup>, TPB<sup>-</sup>, and some LTC<sup>-</sup>-related compounds) inhibited RVD at micromolar concen-

trations 3–20  $\mu\text{M}$ . In contrast, the lipophilic cation tetraphenylphosphonium ( $\text{TPP}^+$ , 10–50  $\mu\text{M}$ ) did not affect RVD in mammalian cells (*data not shown*).

It is interesting to note that despite their widely differing chemical structures, all of the lipophilic anions tested here were found to be very potent RVD blockers acting at low micromolar concentrations (Fig. 3). For comparison, many specific inhibitors of membrane channels (e.g., DIDS, or NPPB) are also capable of abolishing RVD, but usually at much higher concentrations ranging typically between 0.1 and 1 mM [8, 24].

The strong RVD inhibition by a wide range of structurally dissimilar lipophilic anions does not support the idea that the effect of  $\text{LTC}^-$  on volume regulation was due to its specific interaction (e.g., as a channel blocker) with the membrane channels/transporters involved in RVD. It seems more likely that the adsorption of  $\text{LTC}^-$  (and other lipophilic anions) inhibited RVD by modifying the electrostatic potential profile across the asymmetric cell membrane. Electrophysiological studies on cells and artificial lipid bilayers designed to distinguish clearly between the two possible inhibition mechanisms will be subject of future research in our laboratories. Although the mechanisms by which  $\text{LTC}^-$  inhibited RVD is not yet understood, the high  $\sigma_i$  values observed in ROT measurements (Fig. 4B, Table 4) indicated that  $\text{LTC}^-$  prevented the leakage of intracellular ions from hypotonically swollen cells.

### Membrane Charging

As seen from the ROT spectra (Fig. 4B),  $\text{LTC}^-$  gave rise to an additional antifield peak in the ROT spectrum of Jurkat cells. This low-frequency peak implies that the plasma membrane exhibits a strong dielectric dispersion in the kHz range with a magnitude ( $\Delta C_m$ ) which is proportional to the area-specific concentration ( $N_i$ ) of the adsorbed lipophilic anions (mobile charges) [30]:

$$\Delta C_m = \frac{N_i F^2}{2RT} \quad (4)$$

whereby  $F$ ,  $R$  and  $T$  ( $\approx 23^\circ\text{C}$ ) have their usual meanings. The characteristic frequency ( $f_{\text{LTC}}$ ) of the dispersion is related to the translocation rate ( $k_i$ ) of  $\text{LTC}^-$  across the membrane as  $f_{\text{LTC}} = k_i/\pi$ . Using the values  $N_i = 0.63 \text{ pmol/cm}^2$  and  $k_i = 3.7 \times 10^5 \text{ s}^{-1}$  derived from the ROT spectra, a capacitance increment  $\Delta C_m \approx 1.2 \text{ }\mu\text{F/cm}^2$  is obtained at frequencies below  $f_{\text{LTC}} \approx 120 \text{ kHz}$ . This means that doping the cell membrane with  $\text{LTC}^-$  resulted in an about 2.5-fold capacitance increase ( $C_m + \Delta C_m \approx 1.9 \text{ }\mu\text{F/cm}^2$ ) above that of the unmodified membrane in hypotonically swollen Jurkat cells ( $C_m = 0.7 \text{ }\mu\text{F/cm}^2$ , Table 4).

The dependence of  $\tau_m$  on  $C_m$  given by Eq. 2 implies that the relaxation of mobile charges within the cell membrane should reduce the rate of membrane charging. For control and LTC-treated Jurkat cells in sorbitol-substituted pulsing medium, the  $\tau_m$  values of 0.38  $\mu\text{s}$  and 0.63  $\mu\text{s}$ , respectively, were calculated by using the data from Tables 2 and 4 in Eq. 2. Despite slower membrane charging, the electroporation efficiency was apparently not compromised, as suggested by the increased PI uptake (Fig. 6), as well as by the enhanced transfection yield and GFP expression in various mammalian cells after treatment with  $\text{LTC}^-$  (Table 5).

### Cytosolic Polarization and Electrodeformation Force

As pointed out by [13, 32], membrane charging and breakdown are preceded by the electrodeformation force  $F_D$  that arises from the electrostatic interaction between the applied field  $E_0$  and induced cell dipole  $\mu_C$  and is determined by the ratio  $\sigma_i/\sigma_e$  as follows:

$$F_D \propto E_0^2 \frac{(\sigma_i^2 - \sigma_e^2)}{(\sigma_i + 2\sigma_e)^2}. \quad (5)$$

for  $\sigma_e < \sigma_i$ ,  $F_D$  tends to elongate the cell along the applied field  $E_0$ . This effect has been shown to be responsible for the improved electropermeabilization in low-conductivity solutions [20, 32]. The electrodeformation force  $F_D$  arises very fast with the time constant of cytosolic polarization  $\tau_i$ , being inversely related to the peak frequency  $f_{c2}$  of co-field electro-rotation (Fig. 4):

$$\tau_i = \frac{1}{2\pi f_{c2}} = \frac{\epsilon_0(\epsilon_i + 2\epsilon_e)}{\sigma_i + 2\sigma_e}. \quad (6)$$

Using the values for H7 cells ( $\sigma_i = 2.2 \text{ mS/cm}$  and  $\epsilon_i = 58$ ) derived from ROT spectra (Table 4), the  $\tau_i$  value of  $\sim 7.9 \text{ ns}$  was calculated for this cell line suspended in hypotonic fusion solution ( $\sigma_e = 120 \text{ }\mu\text{S/cm}$ ,  $\epsilon_i = 80$ ). As already mentioned, the cytosolic conductivity  $\sigma_i$  in  $\text{LTC}^-$ -treated cells was generally higher than in untreated control (Table 4). According to Eq. 5, an increase of  $\sigma_i$  leads to a stronger electrodeformation stress on the cell membrane, which can explain the improved PI uptake (Fig. 6) and plasmid transfection (Table 5).

Taken together, the beneficial effect of  $\text{LTC}^-$  on the transfection yield and GFP gene expression observed in the present study (Table 5) can be attributed to (1) the increased cell size due to the RVD inhibition, and (2) also to the increased electrodeformation force due to the higher  $\sigma_i$  values (Table 4). The reason for the higher proliferation activity of electrotransfected cells (Table 5, PF data) might be the ability of  $\text{LTC}^-$  to prevent excessive ion losses from the cytosol of hypotonically swollen cells. Whatever the explanation,  $\text{LTC}^-$  seems to be a useful

pulse medium additive which improved the robustness of electromanipulation protocols, rendering them more efficient and less dependent on the duration of hypotonic stress.

## CONCLUDING REMARKS

This study clearly demonstrates that hypotonic sugar-substituted media are useful for the efficient electrotransfection and electrofusion of mammalian cells. We found that in such media the direction and magnitude of the secondary cell volume changes of cells depended strongly on both medium osmolality and its sugar composition. Moreover, the secondary volume responses to hypotonicity varied considerably among different cell types studied here. Therefore, electroporation protocols should be optimized not only for the solution osmolality, but also the duration of hypotonic treatment prior to electropulsing should be carefully synchronized with the transient volume response of the particular cell type. Furthermore, doping the cell membrane with lipophilic anions prevented both secondary cell shrinkage and ion losses from the cytosol (probably due to the inhibition of volume-sensitive channels), which might be the reasons for the improved electroporation efficiency and higher growth rates of transfected cells. A further major enhancement of protocols along with greater consistency of results between different cell batches was achieved after screening a number of growth medium additives for the highest survival rates of electroporated and electrofused hybrid cells. The experimental findings and approaches presented here can generally be used for the development of cell-type-specific electroporation conditions, thus facilitating rational design of electromanipulation protocols, especially for rare and valuable human cells.

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