Efficient *in situ* electroporation of mammalian cells grown on microporous membranes

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Received June 20, 1995; Accepted June 27, 1995

ABSTRACT

Electroporation is a common technique for the introduction of DNA molecules into living cells. The method is currently limited by the necessity of applying the electrical discharge to cells in suspension. Adherent cells must therefore be removed from their substratum, which can induce unwanted physiological effects. We report here a new procedure for *in situ* electroporation of cells grown on microporous membranes of polyethylene terephthalate (PET) or polyester (PE). We demonstrate that this method of *in situ* electroporation employs only readily available materials and standard electroporation devices without any modifications, is as efficient as conventional electroporation of cells in suspension, and is applicable to a wide range of cell types. Efficient electroporation can be achieved under conditions of minimal cell killing, and can be performed with quiescent cells as well as with confluent epithelial sheets. The method is a useful extension of electroporation technology, and will allow the application of electroporation to a wider spectrum of biological systems.

INTRODUCTION

Electroporation involves exposing cells to a short electric pulse, which causes transient pores to form in the cytoplasmic membrane, making it possible for macromolecules to enter (1,2). The method has gained widespread use because of its simplicity, and its ability to introduce DNA into a diverse variety of cell types (3,4). The applicability of electroporation to many biological systems is limited, however, because currently available commercial devices are designed to apply the electrical discharge only to cells in suspension.

The current method of electroporating mammalian cells requires preparation of a cell suspension. Adherent cells must therefore be detached from their substratum using trypsin (or other means). Trypsinization and replating can, however, introduce unwanted physiological perturbations: for example, induce quiescent cells to enter the cell cycle (5,6). Furthermore, electroporation is not a possible method of gene transfer when working with polarized cells or organized cellular structures, such as epithelia, whose architecture would be disrupted by the detachment process.

Recently developed alternatives to electroporation include encapsulation of DNA in cationic liposomes (7) and microprojectile bombardment (8). Both of these methods can be used to introduce DNA into adherent cells while growing *in situ*, but each method has certain limitations. For example, perturbation of the lipid composition of cellular membranes has physiological consequences, including, in some cases, acute toxicity. Microprojectile bombardment is limited by its mechanical trauma as well as the requirement for specialized equipment.

One method of *in situ* electroporation utilizes cells grown on CytodexTM microcarrier beads which can be suspended in a conventional electroporation cuvette (9). This method is rather inefficient and does not lend itself to microscopic examination of the electroporated cells. Another method utilizes cells grown on a glass surface that has been coated with an electrically conductive material (10-12). This method is also limited by the need for specialized equipment. We report here a method of *in situ* electroporation of cells grown on microporous membranes of polyethylene terephthalate (PET) or polyester (PE). The novel feature of this method is that it uses readily available materials (PET or PE membranes) and standard commercial electroporation devices without any modifications.

Many types of cells can be readily grown on a variety of porous membranes (13,14). Membrane-grown cells and cell structures have been used for many types of microscopic and physiological studies, including the study of polar cells and polar events, cell movement, transport, and polarized membrane domains (15-18). Both PET and PE membranes are transparent, which allows study by light microscopy, and do not possess intrinsic fluorescence, which allows the application of immunofluorescence methods without the need to detach the cells from the membranes. We present here an optimization of electronic parameters for *in situ* electroporation of several cell lines and cell strains. We show that the method is reproducible, applicable to a variety of cell types, and, in most cases, equivalent in its efficiency to standard electroporation in suspension.

MATERIALS AND METHODS

Cell culture

TGR-1 is an *hprt*⁻ subclone of the Rat-1 cell line (19). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, containing glutamine, pyruvate and high glucose) supplemented with 10% calf serum (CS). Where indicated, TGR-1 cells were made

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Figure 1. Expression of β -galactosidase in cells electroporated *in situ*. (A) Growing TGR-1 cells. (B) Quiescent TGR-1 cells. (C) Human keratinocytes. In (A), cells were grown in media containing 10% CS and electroporated at 50–70% confluence (25 μ F, 130 V) in DMEM. In (B), cells were made quiescent by serum deprivation prior to electroporation (3 μ F, 90 V) in DMEM. In (C) cells were grown to confluence and held (24–36 h) until the monolayers assumed a uniformly packed, cobblestone-like appearance, then electroporated (50 μ F, 60 V) in MCDB 153. In all cases, cells were attached to Falcon membranes, electroporated with 100 μ g/ml pRSVlacZ, histochemically stained for β -galactosidase activity, and counterstained with crystal violet. Cells were photographed using a Nikon DiaphotTM inverted microscope at 200 $\times$ magnification under brightfield (tungsten) illumination on Kodak EktachromeTM film. Gene Pulser was used in experiments shown in (A) and (B); Gene Pulser II was used in the experiment shown in (C).

quiescent using one of two methods: contact inhibition (incubation under confluent conditions for 72 h without serum starvation), or serum deprivation (incubation at 80% confluent conditions for 36–48 h in 0.25% serum). CHO (ATCC CCL 61), HeLa (ATCC CCL 2), COS-7 (ATCC CRL 1651) and MRC-5 (ATCC CCL 171) cells were obtained from the ATCC (Rockville, MD). FF (fetal fibroblast) cells were derived from a human fetus and obtained from Dr R. Halaban, Department of Dermatology, Yale University. FF cells were between passage numbers 5 and 15. CHO, HeLa and FF cells were cultured in Ham's F-12 medium; COS-7 and MRC-5 cells were cultured in DMEM. All media were obtained from Life Technologies, Inc. CHO, HeLa, COS-7 and MRC-5 cells were grown in media supplemented with 10% fetal bovine serum (FBS); FF cells were supplemented with 15% FBS. Human keratinocytes were prepared from neonatal foreskins and cultured in serum-free low calcium MCDB 153 medium, as described (20).

Electroporation techniques

For *in situ* electroporation, tissue culture-treated membranes from Falcon, Inc. (0.45 μ m pore Cell Well InsertsTM, 1×10^6 pores/cm², catalog #3090) or Costar, Inc. (0.45 μ m pore Transwell Clear InsertsTM, 4×10^6 pores/cm², catalog #3450) were cut into 0.8 $\times$ 2.0 cm strips, sterilized in ethanol, transferred to a 35 mm culture dish (or a well of a 6-well cluster dish), and washed with 1–3 ml of media. Trypsinized cells in culture medium were added to the wells and allowed to attach to the membranes during an overnight incubation. The following day, using sterilized, fine-point forceps, the membrane strips were transferred into 0.4 cm cuvettes (Bio-Rad, Inc.) filled to the top with electroporation medium and were allowed to settle between the electrodes. The membranes were inserted with the cells facing the negative electrode. The electroporation medium was either HEPES-buffered saline (HBS; ref. 21), DMEM medium or Opti-MEMTM medium (Life Technologies, Inc.), as indicated, in the absence of serum. The electroporation medium was carefully removed by aspiration with a Pasteur pipette and replaced with 0.5 ml of medium containing the indicated amount of DNA. This results in submersion of the lower 13 mm of the membrane in the electroporation medium. The cells that are above the surface of the electroporation medium do not experience the electric discharge, and serve as convenient controls for the effects of handling procedures other than the electroporation. Electroporations were performed with a Bio-Rad, Inc. Gene PulserTM or Gene Pulser IITM, and Capacitance ExtenderTM. After electroporation, the membranes were left in the cuvettes for 2–10 min before being placed back in culture medium. The number of cells that were actually electroporated on the membranes was determined by harvesting, at the time of electroporation, duplicate 35 mm plates inoculated without membranes, counting the cells in a hemocytometer, and calculating the cell density (corrected for the surface area of the submerged portion of the membrane). Where indicated, cell viability was assessed with 0.4% trypan blue and light microscopic observation 10–15 min later. For standard (in suspension) electroporation, cells at 50–75% confluence in 75 cm² flasks were trypsinized, pelleted, resuspended in Opti-MEM medium to remove residual serum, and re-pelleted. The cells were resuspended at 5×10^6 to 1×10^7 cells/ml in Opti-MEM medium, an aliquot of the cell suspension was mixed with plasmid DNA, and 0.1 ml was electroporated in a 0.2 cm cuvette using the Gene Pulser II. All electroporation operations were performed at room temperature.

Plasmids and expression assays

The plasmids pH β Luc (22), pCMV-Luc (23) and pSV2ALAA Δ 5' (24) are mammalian expression vectors which express the firefly luciferase gene from the human β -actin promoter, the cytomegalovirus early promoter, or the SV40 early promoter, respectively. Plasmid pRSVlacZ (25) contains the *Escherichia coli* β -galactosidase gene expressed from the RSV LTR. Luciferase activity was assayed in cell extracts prepared by detergent lysis 24 h post-electroporation as described by Heiser (8). In all cases, luciferase activity is expressed in relative light units (RLU) standardized against purified firefly luciferase (Analytical Luminescence Laboratory, Inc.). β -galactosidase activity was assayed *in situ* by histochemical staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) 48 h after electroporation as described by Herzing and Meyn (26). The membrane strips were counterstained with crystal violet, rinsed in water, and mounted in 80% glycerol under a cover slip sealed with clear nail polish.

[³H]thymidine incorporation

Where indicated, [methyl-³H]thymidine (85 Ci/mmol) was included in culture medium at a concentration of 1.5 μ Ci/ml. Membrane strips were fixed with 100% methanol and air dried. (Samples can also be stained with X-gal as indicated above, dehydrated in graded ethanol washes [50, 70, 80, 90 and 100% EtOH, 15 s each wash], and air dried, prior to application of emulsion.) Dried membrane strips were attached along one of their short edges to a 3 $\times$ 1" glass microscope slide (cells facing outward) using yellow electrophoresis tape. Slides with the mounted membranes were dipped in Kodak, Inc. NTB2TM emulsion (diluted 1:1 with water) and exposed (at 4°C) in a light-tight box containing desiccant. Slides were developed in Kodak DektolTM developer (diluted 1:1 with water, 2 min at 15°C) and fixed in Kodak fixer (5 min at 15°C). The membranes were finally counterstained and mounted for microscopy (as described above). To process samples for scintillation counting, 100% methanol-fixed membranes were incubated in two changes (30 min each) of 5% trichloroacetic acid (TCA) at 4°C. Cells were dissolved in 0.5 ml of 1 M NaOH for 1 h at 60°C. The samples were neutralized with glacial acetic acid and counted using Packard, Inc. Ultima GoldTM scintillation cocktail.

RESULTS

Optimization of *in situ* electroporation conditions in dividing and quiescent cells

Maximum transformation by electroporation is typically obtained in the range of 20–50% cell killing (1,27). Electroporation conditions for TGR-1 cells in suspension have previously been optimized (19); these same conditions resulted in complete (100%) killing when membrane-attached cells were electroporated. The majority (>99%) of cells were completely disintegrated 15 min after electroporation, and the few cells still attached to the membrane were stained by trypan blue (data not shown). This result shows that the electroporation environment is very different for adherent and suspension cells. Similar results were found with CHO, COS-7 and MRC-5 cells (data not shown).

Suitable electroporation conditions for adherent cells were systematically explored utilizing the whole capacitance range of the Gene Pulser; a range of voltages (100–450 V) were tried. Following electroporation, cells were placed in culture medium and examined microscopically after 15–30 min. Samples that showed high cell disintegration (>50%) were stained with trypan blue. Samples that showed acceptable levels of cell disintegration (<50%) were incubated overnight, re-examined microscopically, then stained with trypan blue. We found that cell disintegration was very rapid and essentially complete after 15 min incubation, but cell death progressed significantly during the overnight incubation. Very little (if any) cell death occurred afterwards. The highest field strengths without excessive cell death were achieved at the settings of 25 μ F and 100–130 V (Table 1).

Table 1. Fine-tuning of electronic parameters for cell viability

Voltage (V)	Time constant (ms)	Cells remaining on membrane top (%)	Cells remaining on membrane bottom (%)	Dead cells (%)
70 (80)	1.5	100	100	<5
100(110)	1.4	100	100	<5
130(140)	1.4	100	60	5
160(160)	1.3	80	40	5
190(200)	1.2	90	<5	5
220(230)	1.3	80	25	10
250(260)	1.2	60	10	10
280(280)	1.2	60	<5	10

Cells (TGR-1) on the membranes were 50–70% confluent at the time of electroporation. DMEM was used as the electroporation medium. Capacitance was held at 25 μ F. The values in parentheses are the actual voltages delivered by the Gene Pulser. It should be noted that in some cases the delivered voltage was slightly higher than the set voltage. These discrepancies were always of small magnitude, and occurred sporadically at low capacitance settings (25, 3 and 1 μ F). After electroporation, cells were incubated for 12 h in culture medium. Cells remaining on membranes were assessed by microscopic observation and are given as the percent of cells present on the unelectroporated portion of the membrane. It was noted that there was a gradient of cell killing from the top to the bottom of the membrane. Cell killing was also greater along the edges of the membranes. Values for dead cells are trypan blue-stained cells expressed as percent of total cells remaining on the electroporated portion of the membrane. Except at high voltages, essentially all the cells present on the membranes 12 h after electroporation excluded trypan blue. The Gene Pulser and Falcon membranes were used in this experiment.

Electroporation of quiescent TGR-1 cells was also investigated. Quiescence was induced by two methods: serum deprivation or contact inhibition. Serum starvation is significantly more effective in synchronizing cells in the G₀ phase than is simple contact inhibition (the two methods yield >95% and 80–85% of cells with diploid DNA content, respectively; ref. 25). The electroporation conditions optimized for exponentially growing cells (25 μ F, 100 V) were also found to be optimal for cells made quiescent by contact inhibition in 10% serum (data not shown). However, cells made quiescent by serum deprivation were much more sensitive to electroporation-induced killing (Table 2). The optimum conditions were determined to be 3 μ F and 70–100 V. Electroporation in HBS rather than DMEM generally resulted in somewhat less killing (data not shown).

Figure 2. Effect of electroporation on DNA synthesis in TGR-1 cells measured by *in situ* autoradiography. (A) Quiescent cells. (B) Quiescent cells, serum stimulated. (C) Quiescent cells, electroporated. Cells attached to Falcon membranes were made quiescent by serum deprivation, labelled with [3 H]thymidine, and processed for autoradiography as indicated (Materials and Methods). Cells were labelled during the period of 12–24 h following mock (A), serum (B) or electroporation (C) treatment. Electroporation with Gene Pulser (C) was in DMEM in the absence of DNA (3 μ F, 90 V). Cells were autoradiographed for 72 h at 4°C then photographed using a Nikon Diaphot inverted microscope at 100 $\times$ magnification under phase contrast illumination on Kodak T-Max400TM film.

Table 2. Optimization of electronic parameters for quiescent (serum-starved) cells

Capacitance (μ F)	Voltage (V)	Time constant (ms)	Cells remaining on membrane		Dead cells (%)
			top (%)	bottom (%)	
25	100(100)	1.7	50	<5	50
3	60(60)	0.4	100	100	<5
3	100(110)	0.3	100	100 ^a	<5
3	150(160)	0.2	100	30	5

Cells (TGR-1) were plated on membrane strips and made quiescent by serum starvation (Materials and Methods). Cells were electroporated as indicated in Table 1. The voltage values in parentheses are the actual voltages delivered by the Gene Pulser. Cells remaining on membranes and dead cells were scored as indicated in Table 1. The Gene Pulser and Falcon membranes were used in this experiment.

^aExcept along edges.

***In situ* electroporation results in very efficient transfection of DNA**

To determine the efficiency of *in situ* electroporation, TGR-1 cells were electroporated under optimal conditions with 100 μ g/ml pRSVlacZ plasmid DNA and histochemically stained for β -galactosidase. Approximately 20–30% of exponentially growing cells expressed β -galactosidase activity 48 h post-electroporation (Table 3 and Fig. 1A). β -galactosidase expression was not entirely uniform across the membranes, and discrete areas with up to 40% β -galactosidase-positive cells were evident. Electroporation of contact inhibited cells was somewhat less efficient, with 10–15% of the cells expressing β -galactosidase, while electroporation of serum-starved cells was significantly less efficient, in the range of 1–3% β -galactosidase-positive cells (Fig. 1B). In comparison, electroporation of exponentially growing cells by the standard (in

suspension) technique under optimal conditions resulted in an efficiency of 28% (data not shown).

Stimulation of DNA synthesis by electroporation of quiescent cells

Although it has been reported that electroporation does not perturb quiescence (10), we re-investigated cell cycle entry under our *in situ* electroporation conditions. Cells on membranes rendered quiescent by serum deprivation were electroporated, and subsequently incubated for 12 h in low serum medium. [3 H]thymidine was added to the medium and incubation was continued for 12 h. The membranes were then fixed and autoradiographed (Materials and Methods). As controls, unelectroporated cells were either kept continuously in low serum (negative control), or stimulated with 10% serum at the time of electroporation (positive control). The positive and negative control samples displayed ~95 and 5%, respectively, of nuclei overlaid with silver grains (Fig. 2). The electroporated sample displayed 30–40% of nuclei overlaid with silver grains.

Two lines of evidence indicate that the increased labelling index in the electroporated cells does not represent physiological entry into S phase. First, it was noticed that the density of the silver grains was much lighter over electroporated and negative control samples than over positive control samples (Fig. 2). When autoradiography was performed for only 24 h, the electroporated and negative control samples showed essentially no labelling, while the signal in the positive control samples remained strong (data not shown). Secondly, the experiment was repeated under identical conditions, except that the membranes were processed for scintillation counting instead of autoradiography. The results showed that both the electroporated and negative control samples had equivalent, very low amounts of incorporation (Fig. 3). When electroporation was performed in the presence of pRSVlacZ plasmid DNA there was no correlation between uptake and expression of the DNA and the low level of labelling observed by autoradiography (data not shown).

Table 3. Transfection of *lacZ*-expressing plasmid DNA

Capitance (μ F)	Voltage (V)	Time constant (ms)	Cells remaining on membrane		β -galactosidase positive cells (%)
			top (%)	bottom (%)	
25	70(80)	1.9	100	70	20
25	80(90)	1.9	100	90	25
25	90(90)	2.1	100	50	15
25	100(110)	1.7	100	25	10
3	100(110)	0.3	100	100	3
3	150(160)	0.2	100	30	1

Cells (TGR-1) were made quiescent by contact inhibition (25 μ F electroporations), or serum starvation (3 μ F electroporations). Cells were electroporated (as indicated in Table 1) in 0.5 ml DMEM containing 50 μ g of plasmid pRSVlacZ. Plasmid DNA was used in supercoiled form, and carrier DNA was not used. After electroporation, the membranes were returned to the spent medium in which they were rendered quiescent, and incubation was continued at 37°C for 48 h. Cells were stained for β -galactosidase activity and unelectroporated cells were counterstained with crystal violet (Materials and Methods). The voltage values in parentheses are the actual voltages delivered by the Gene Pulser (see Table 1). Cells remaining on membranes were scored as indicated in Table 1. β -galactosidase-positive cells were scored by microscopic observation under bright-field illumination. The values are expressed as percent of total cells remaining on the electroporated portion of the membrane. The Gene Pulser and Falcon membranes were used in this experiment.

In situ electroporation of diverse cell types

We found that *in situ* electroporation can be successfully applied to many different cell lines and cell strains. In addition, a side-by-side comparison of standard and *in situ* electroporation techniques showed that the two methods are in most instances of comparable efficiency (Table 4). The cell lines employed in these experiments were CHO, HeLa and COS-7; we also employed the human fibroblast cell strain MRC-5, and recently explanted human fetal fibroblasts (FF). To allow the processing of the large number of samples required in these experiments we utilized assays of transient expression of luciferase-encoding vectors. Luciferase enzymatic activity was normalized to the number of cells present in the samples at the time of electroporation.

The results showed that electroporation of CHO and COS-7 cells by the two techniques was equally efficient (Table 4). Likewise, electroporation of TGR-1 rat fibroblasts by the two techniques was of very similar efficiency (above). Electroporation in suspension was ~3-fold more efficient for HeLa cells, and for human fibroblast cell strains this difference increased to ~10-fold. We believe that the reason for the large differences in electroporation efficiencies with human fibroblasts is their high sensitivity to contact inhibition. We have shown (above) that for TGR-1 rat fibroblasts *in situ* electroporation efficiencies decrease dramatically with increasing quiescence. In the experiments presented in Table 4 all *in situ* electroporations were performed at 70–80% confluency, whereas standard electroporations employed exponentially growing cells.

The electroporations presented in Table 4 were performed under optimal conditions; a typical optimization experiment for one cell line (CHO) is shown in Figure 4. It can be seen that different combinations of electronic parameters can be employed (e.g., high voltage/low capacitance, or low voltage/high capacitance), but that under a given set of conditions the optimum efficiency occurs in a rather narrow range. Furthermore, we have observed that best *in situ* efficiencies are obtained with the low voltage/high capacitance combination, with the optimum in the range of 100–120 V.

The electroporations presented in Table 4 were performed in the linear range of the DNA concentration dose–response curve (Fig. 5). It can be seen that for CHO cells, an increase in DNA concentration from 0.2 to 40 μ g/ml results in a linear increase in

Figure 3. Effect of electroporation on DNA synthesis in quiescent TGR-1 cells measured by total [³H]thymidine incorporation. TGR-1 cells attached to Falcon membranes were treated as in Figure 2, except that membranes were processed for scintillation counting (Materials and Methods). Column 1: quiescent cells; column 2: quiescent cells, serum-stimulated; column 3: quiescent cells electroporated in DMEM; column 4: quiescent cells electroporated in HBS. To eliminate artifacts due to loss of cells either during electroporation or subsequent handling, the entire membranes were examined microscopically immediately prior to the dissolution of cells for scintillation counting; in (1) and (2), cells were confluent over the entire membrane surface; in (3) and (4), no portion of the electroporated membrane surface had cells below 80% confluent density. The raw counts were (in c.p.m.): column 1, 4.2×10^4 ; column 2, 9.1×10^5 ; column 3, 3.5×10^4 ; column 4, 2.6×10^4 . The Gene Pulser was used in this experiment.

electroporation efficiency, and that throughout this range the standard and *in situ* techniques produce completely parallel efficiency values. Analogous results were observed with other cell types (data not shown). The linear portion of the DNA concentration dose–response curve extends beyond 100 μ g/ml, and for some cell lines, such as TGR-1, up to 200 μ g/ml (data not shown). However, unless explicitly demanded by the experimental setting, such very high DNA concentrations are prohibitively expensive.

Table 4. Comparison of standard (in suspension) and *in situ* electroporation efficiencies for diverse cell types

Cell line	DNA (μ g)	Electroporation conditions		Luciferase activity (RLU/ 10^5 cells)	
		Attached	Suspension	Attached	Suspension
CHO	pH β Luc (10)	450 μ F, 60 V	500 μ F, 130 V	2.2×10^5	2.4×10^5
COS-7	pSV2ALA Δ 5 (20)	200 μ F, 60 V	500 μ F, 160 V	4.4×10^7	3.1×10^7
HeLa	pCMV-Luc (20)	1000 μ F, 120 V	500 μ F, 160 V	2.6×10^6	9.7×10^6
FF	pCMV-Luc (10)	100 μ F, 50 V	500 μ F, 140 V	0.6×10^5	7.0×10^5
MRC-5	pCMV-Luc (10)	100 μ F, 60 V	1000 μ F, 250 V	1.3×10^5	1.5×10^6

The indicated cell lines or cell strains were cultured as described in Materials and Methods. Cells were 70–80% confluent on the membranes at the time of electroporation. For standard electroporation cells were harvested at 40–50% confluence. The electroporation medium was Opti-MEM. Electroporations in suspension were performed in 0.2 cm gap cuvettes with 0.1 ml electroporation medium; electroporations *in situ* were performed in 0.4 cm gap cuvettes with 0.5 ml electroporation medium. After electroporation, cells were incubated in complete culture medium for 24 h. For *in situ* electroporations, extracts were prepared by rinsing the membrane strip several times in PBS and vortexing with 0.2 ml of lysis buffer in a microfuge tube. Gene Pulser II was used in all experiments; Falcon membranes were used with CHO and COS-7 cells, and Costar membranes were used with HeLa, FF and MRC-5 cells.

Figure 4. Optimization of *in situ* electroporation conditions measured by transient expression of luciferase. CHO cells attached to Falcon membranes were electroporated in Opti-MEM containing 4 μ g/ml pH β Luc using Gene Pulser II. Cells were harvested 24 h post-electroporation and assayed for luciferase activity. In this experiment, $\sim 1.9 \times 10^5$ cells were present on the submerged (electroporated) portion of the membrane. Each value is the average of two electroporations.

***In situ* electroporation of organized epithelial cell structures**

Human keratinocytes were prepared from neonatal foreskins and cultured under low calcium conditions in serum-free medium supplemented with defined growth factors. This culture system was developed specifically to maintain keratinocytes in an undifferentiated state, as evidenced by, for example, their small size and the absence of differentiation markers (20). When keratinocytes reach confluence under these culture conditions, they form an organized, single-cell epithelial sheet, but fail to express markers of differentiation (28). Addition of calcium elicits the rapid formation of desmosomes, and subsequently of a multilayered, stratified epithelium, which contains highly differentiated cells.

We found that undifferentiated keratinocytes could be grown into a single-cell epithelial sheet on microporous membranes, and subsequently electroporated *in situ* (Fig. 1C). The electroporation efficiency was low (0.1–0.5%), but damage to the monolayer was minimal; the cells maintained a healthy, cobblestone-like appearance, as evidenced by counterstaining with crystal violet. It is noteworthy that keratinocytes are known to be one cell type especially refractory to electroporation (29,30). Furthermore, following electroporation the single-cell epithelium could be triggered to stratify by the addition of calcium (data not shown). *In situ* electroporation is thus a promising method of introducing exogenous DNA into organized cell structures with minimal physiological perturbations.

DISCUSSION

We have found that adherent mammalian cells grown on microporous membrane supports can be subjected to *in situ* electroporation simply by placing the membrane between the electrodes of a commercially available electroporator. We present here a systematic investigation of parameters for *in situ* electroporation, as well as a comparison of the standard (in suspension) and *in situ* electroporation techniques for several commonly employed cell lines and cell strains. We demonstrate that in terms of efficiency the two methods are, in most instances, equivalent.

We have used membranes of polyethylene terephthalate (PET) or polyester (PE), but it is likely that other materials, such as polycarbonate, as long as they are porous and can support adequate cell growth, could also be employed. We have used membranes with 0.45 μ m pores, and a pore density of 1×10^6 (Falcon) or 4×10^6 (Costar) pores/cm². In both cases, each cell straddles several pores. We have found that optimum electroporation of cells attached to Costar membranes occurs at slightly lower electric fields than of cells attached to Falcon membranes (data not shown). Using the same electronic parameters, the higher pore density of the Costar membranes results in less resistance between the electrodes and hence a lower time constant (τ). In practical terms, we have found that expression in cells electroporated under optimal conditions on Costar membranes is slightly ($\sim 20\%$), but consistently, higher than in cells electroporated on Falcon membranes. We have also investigated membranes containing pores with larger diameters. We have not seen any consistent differences in expression levels between cells

Figure 5. The influence of DNA concentration on electroporation efficiency for standard and *in situ* electroporation. CHO cells attached to Costar membranes were electroporated in the presence of the indicated concentrations of pH β Luc. Standard electroporations were performed in Opti-MEM at 500 μ F and 130 V; electroporations *in situ* were performed in Opti-MEM at 400 μ F and 60 V. In both cases Gene Pulser II was used. Each value is the average of three determinations; the bars show the standard errors. The insert shows the data replotted using linear X and Y axes to emphasize the linearity of the DNA concentration dose-response curve.

electroporated on 0.45 and 3.0 μ m pore membranes, however, the larger pores interfere significantly with microscopy.

Our current *in situ* electroporation technique involves cutting the membranes manually to the approximate dimensions of a 0.4 cm electroporation cuvette (0.8 $\times$ 2.0 cm) so that they can be inserted between the electrodes with sterile forceps. One limitation is that the membrane must be rigid enough not to spontaneously curl, but flexible enough that it does not crack. Another limitation is that, due to the flexibility of the membranes, the precise position of the membrane in the cuvette, and thus the distance of the membrane from either electrode, cannot be controlled. We have observed some variability from experiment to experiment, and also between different portions of the same membrane. We believe this is due to the local heterogeneities in the electric field caused by the imprecision of membrane positioning. We are currently developing a rigid frame to hold the membrane stretched at a precisely fixed position in the cuvette.

When the membrane is oriented in the cuvette such that the cells face the cathode (negative electrode), expression is at least 5-fold higher than when the cells are facing the anode (data not shown). This orientation results in the DNA being driven into the cells through their exposed surface. As electroporation medium we have investigated HEPES-buffered saline (HBS), and four different culture media formulations (DMEM, Opti-MEM, F10, and MCDB 153), either with or without serum supplementation, and we did not observe significant and consistent differences in electroporation efficiency. We therefore recommend using basal medium (without serum or growth factors) in which the cells are normally cultured as the electroporation medium. In a few cases it has been reported that inclusion of serum can increase electroporation efficiency (31), we believe this may be due to an

enhancement of cell survival (allowing the application of higher electric fields), and is probably a cell-line specific phenomenon (in this situation serum should be heat-treated to inactivate nucleases).

We have consistently observed, with all the cell types investigated, that maximum electroporation efficiency *in situ* occurs at ~80% cell survival, whereas maximum efficiency in suspension occurs at ~20% cell survival. This was observed in assays using both the luciferase reporter (which measures the total activity elicited in the population of cells) and the β -galactosidase reporter (which measures the fraction of the cells that become electroporated). We believe this is because *in situ* cell killing increases very rapidly and becomes the dominant factor affecting the yield of electroporated cells. Raptis and Firth (10) reported consistent results with the F111 cell line.

During standard electroporation the cells act as dielectric spheres, and the voltage at the cell surface is proportional to the applied electric field and the radius of the cell (32). The cell membrane acts as a resistor, and above a critical field strength localized breakdown occurs which leads to the formation of pores. All membrane sites experience the same field, and a large fraction of the electric discharge can flow around the cells through the medium. In contrast, during *in situ* electroporation, the microporous membrane acts as a resistor, and the electric discharge can only occur through the pores. Although the electrophysical forces operative during *in situ* electroporation have not been investigated in detail, it is clear that the cells experience a very different environment from that during electroporation in suspension. For example, it is apparent that the electrical field is distributed very unevenly over a cell during *in situ* electroporation, with only patches of membrane (the area directly above the pores) likely to experience intense field strengths. We believe, therefore, that it is the focusing of the electric field through the pores of the microporous membrane that can account for the relatively very gentle electronic parameters required for *in situ* electroporation, as well as for the acute sensitivity of the cells to increases in field strength, and the close dependence of optimum electronic parameters on pore size and density.

In the fraction of the cells that survive *in situ* electroporation (which, under conditions of maximum efficiency is the majority of the population) physiological perturbations appear to be minimal. We have shown that *in situ* electroporation does not cause entry into the cell cycle; in contrast, trypsinization and re-seeding is known to cause cell cycle entry in some cell types (5,6). The ability to introduce exogenous DNA (or other macromolecules) into quiescent cells by *in situ* electroporation should be a valuable tool in studies of cell cycle regulation. The efficiency of electroporation into quiescent cells is significantly lower than into exponentially growing cells, but it should be adequate under most experimental settings. For example, as the average total number of cells that can be electroporated on a single membrane is $1-2 \times 10^5$, even a very low efficiency of 0.1% would result in 100-200 independent electroporation events.

Another important application of *in situ* electroporation is the introduction of macromolecules into organized cellular structures. We have demonstrated here the possibility of introducing DNA into epithelial sheets of human keratinocytes. For these applications, standard electroporation is not a possibility, because it would disrupt specialized features, such as functional junctions

between cells, and thus disrupt the integrity of the organized structure.

ACKNOWLEDGEMENTS

We acknowledge Mary Shramke for suggesting the idea of cutting membranes into strips so that they could be fitted into an electroporation cuvette. We thank Leonard Milstone and the members of his laboratory for providing human keratinocytes, and for advice concerning their culture. We thank Philippe Male for expert photography. This work was supported by NIH Grant GM-R01-41690 and a Presidential Young Investigator Award DMB-9057715 from the National Science Foundation to J.M.S.

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