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Electroporation-mediated delivery of molecules to model intestinal epithelia

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Abstract

This study was conducted to determine if electroporation can deliver membrane-impermeant molecules intracellularly to intact, physiologically competent monolayers that mimic the intestinal epithelium. In addition, the long-term effects of electroporation on these monolayers were studied to determine the kinetics with which monolayers recover barrier function. Caco-2 and T84 cells were electroporated as monolayers using calcein and fluorescein-labeled bovine serum albumin as marker molecules for measuring delivery into cells. Confocal microscopy and flow cytometry were used, respectively, to visualize and quantify uptake of these molecules. Transepithelial resistance was used as a measure of physiologic barrier function. We found that intracellular uptake of calcein and bovine serum albumin occurred uniformly throughout both types of model epithelia and increased as a function of voltage, pulse length, and pulse number. There was no significant difference in uptake resulting from single and multiple pulses of the same total exposure time. We also observed that monolayers exposed to electroporation that induced uptake of up to 10^6 molecules/cell were able to recover normal barrier function within one day. These findings suggest that electroporation may be useful for intracellular delivery into monolayers to study epithelial biology and, possibly, for drug delivery to intestinal epithelium.
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Keywords: Electroporation; Drug delivery; Intestinal epithelium; Monolayer; Caco-2; T84

1. Introduction

Diseases of the gastrointestinal tract could be better studied in vitro and treated in vivo by local intracellular delivery of drugs, proteins, or genetic material. We propose that electroporation could be used to transiently disrupt the plasma membranes of intestinal epithelial cells and deliver molecules intracellularly. This approach might provide a means to improve in vitro models of the intestinal epithelium by, for example, allowing researchers to introduce molecules of interest into intestinal cells to either elicit a particular response or test their effects on cellular functions. Previous efforts to introduce molecules into adherent epithelial cells required cell-by-cell microinjection

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(Yaron et al., 1997). A rapid treatment of the whole monolayer by electroporation could provide a more effective approach.

Electroporation might also be useful in vivo for targeted, localized drug delivery to the intestine for the treatment of inflammatory and other intestinal disorders. Greater efficacy and reduced side effects should result from targeted treatment of the intestinal epithelium, where inflammation occurs (Sands, 2000). A minimally invasive approach could be used, whereby an electroporation probe is introduced rectally and brought in contact with intestinal epithelium in a manner similar to endoscopic exams, biopsies and other procedures commonly used in clinical practice. This method would probably be better suited for treating the lower GI tract, but other minimally invasive approaches might also be possible for treating the upper GI tract. Studies to develop and test such a clinical device are beyond the scope of this study.

Caco-2 and T84 monolayers were selected for this study to model the structure and function of in vivo intestinal epithelium, which is characterized by apical–basolateral polarization with secretory and transport systems and absorptive microvilli on the apical (lumenal) surface, intercellular junctional structures on the lateral membrane surfaces, and additional secretory and transport systems on the basal (submucosal) surface (Shaw, 1996). These cell lines, both derived from human colon carcinoma, are used extensively to study the pathophysiology of the intestinal tract (Dharmsathaphorn and Madara, 1990; Madara, 1997; Artursson et al., 2001).

Electroporation involves the application of short electric pulses that transiently disrupt cell membranes and thereby transfect cells with exogenous molecules, such as proteins or genes (Chang et al., 1992). Although the exact molecular mechanism of how electroporation affects the cell membrane is not fully understood, the phenomenon has been widely employed as a research tool and, more recently, as a clinical treatment for some types of cancer (Heller et al., 1999). In most electroporation studies, however, cells have been electroporated in suspension, which poorly mimics in vivo cell function and geometry found in tissues.

Studies of electroporation of cells in monolayers (Liang et al., 1988; Kwee et al., 1990, 1992; Zheng and Chang, 1991; Yang et al., 1995; Wegener et al., 2002), have involved cells that were not epithelial, did not originate from the intestine, or were not of human origin, which limits their ability to model human intestinal tissue function. In related studies (Leonard et al., 2000a,b) used iontophoresis to enhance transport across intestinal epithelium, but did not employ electroporation and, therefore, did not deliver molecules into cells.

In this study, we sought to develop methods for electroporation of in vitro models of intestinal epithelium and determine if large numbers of molecules could be delivered into epithelial cells, while maintaining cell viability. We hypothesized that (1) electroporation can deliver small molecules and macromolecules in a manner that is uniform in both spatial distribution and intracellular concentration and (2) loss of viability and tissue barrier function caused by electroporation can recover rapidly.

To test these hypotheses, we exposed polarized Caco-2 and T84 epithelial monolayers to electrical pulses over a range of different voltages, pulse lengths, and pulse numbers. We then quantified levels of cell viability and uptake of two model compounds: calcein, which served as a model for small, membrane-impermeant drugs, and fluorescein-labeled bovine serum albumin, which modeled macromolecular proteins. To assess long-term effects on epithelial structure and function, we measured the kinetics with which monolayers regained physiologic barrier integrity after electroporation. Finally, we used these results to identify electroporation conditions that can uniformly deliver exogenous molecules into polarized epithelium, while minimizing damage to cells.

2. Materials and methods

2.1. Cell culture

Caco-2 cells (American Type Culture Collection, Manassas, VA) were cultured using standard complete media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer (Mediatech, Herndon, VA), and 0.1 mM non-essential amino acids. T84 cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 nutrient mixture supplemented with 6% (v/v) newborn calf serum,

15 mM HEPES buffer, 14 mM $NaHCO_3$, and antibiotics (40 μg/ml penicillin, 8 μg/ml ampicillin, and 90 μg/ml streptomycin). Unless otherwise stated, all media ingredients were obtained from Invitrogen (Carlsbad, CA). Both cell types were cultured in a 37 °C, 5% CO_2 environment and were passaged using standard cell culture techniques (Dharmsathaphorn and Madara, 1990; Madara et al., 1992).

2.2. Monolayer culture

To grow intact, polarized monolayers, harvested cells were seeded onto collagen-coated Transwell microporous cell culture inserts (Corning Costar, Acton, MA) using well-established techniques (Madara et al., 1992). Caco-2 cells were seeded onto membrane inserts with a growth area of 4.7 cm^2 and a pore size of 0.4 μm (Costar #3450). T84 cells were seeded onto inserts with a growth area of 4.7 cm^2 and pore size of 3 μm (Costar #3414). Both cell lines were incubated in a 5% CO_2, 37 °C environment in their respective growth media and allowed to grow to confluence. Caco-2 monolayers were allowed to remain in culture for 14–21 days, while T84 monolayers were cultured for 7–14 days. Spent media was replaced with fresh media approximately every 48 h.

2.3. Transepithelial resistance measurements

To ensure monolayer integrity, the transepithelial electrical resistance (TEER) of each monolayer was measured using a Millicell ERS apparatus (Millipore, Bedford, MA) prior to each experiment. Resistivity values of 500–600 Ω cm^2 for Caco-2 monolayers (Artursson et al., 1996) and 1500–2000 Ω cm^2 for T84 monolayers (Dharmsathaphorn and Madara, 1990) are considered normal, and were used to indicate the health and confluence of the monolayers. Monolayers with lower than normal resistances were considered to be unable to maintain proper physiology and were not used in these studies. Prior to electroporation, monolayers were rinsed with warm Hanks' balanced salts solution (HBSS) with Ca^{2+} and Mg^{2+} (Sigma Chemical, St. Louis, MO) supplemented with 10 mM HEPES and then placed in fresh electroporation medium (serum-free DMEM buffered with 25 mM HEPES). The presence of calcium in the rinse solution is necessary to maintain the integrity of monolayer tight junctions (Gonzalez-Mariscal et al., 1990). Since the rinse step could affect the resistance, the monolayers were placed back at 37 °C for 15–20 min until resistance recovered to within 10% of initial values.

2.4. Electroporation

Electroporation was carried out using a high voltage pulser (BTX ElectroCell Manipulator 600, Genetronics, San Diego, CA) and an adherent-cell cuvette with parallel, 4-mm gap, aluminum electrodes (InSitu Electroporation System, Thermo Hybaid, Middlesex, UK). The pulser supplied exponential-decay pulses of different voltages and pulse lengths. The inserts on which the monolayers were cultured fit inside the cuvettes as illustrated in Fig. 1.

Two molecules were used to measure delivery to cells: calcein (623 Da, 0.6 nm radius) and fluorescein-labeled bovine serum albumin (BSA; 66,000 Da, 3.5 nm radius) (Molecular Probes, Eugene, OR). Both molecules are membrane-impermeant and fluoresce green when excited at 488 nm. A solution of one of the molecules was added to the electroporation medium on the apical (upper) side of a monolayer at a concentration of 100 μM for calcein and 10 μM for BSA. The monolayers were incubated for approximately 5 min with the fluorescent molecules on the apical aspect to model lumenal administration of

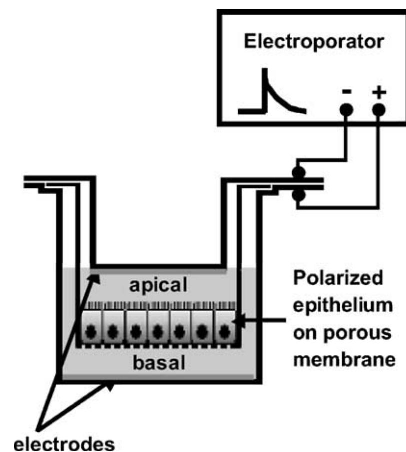

Fig. 1. Diagram of polarized epithelial monolayer grown on a microporous membrane inside an electroporation cuvette. Electrodes located above and below the monolayer were connected to an electroporation pulse apparatus.

drugs. The monolayer was then placed in a cuvette with HEPES-buffered, serum-free DMEM on the basal (lower) side.

Exponential decay electric pulses were delivered at room temperature for voltages ranging from 30 to 400 V, pulse lengths ranging from 1 to 20 ms, and pulse numbers ranging from 1 to 20 pulses. An oscilloscope (HP54062B, Hewlett Packard, Colorado Springs, CO) was used to measure the applied voltages and pulse lengths. In this study, the voltages are reported as the voltage applied to the electroporation cuvette. Voltages across the monolayer are expected to be much less, but were not quantified due to measurement difficulties. The pulse length was measured as the time constant, τ, of the exponential decay pulse. Control monolayers were treated in the same manner, but were not pulsed. Immediately after pulsing, the monolayer was placed in a 37 °C dry incubator with warm DMEM on the basolateral side to recover for at least 15 min and was then incubated further until experiments with other monolayers were completed ($\leq$1 h).

2.5. Dissociation of monolayers for flow cytometry

After electroporation and subsequent cell recovery, each monolayer was washed in warmed HBSS (with calcium and magnesium) to remove media and free calcein or BSA. A 0.5 ml solution containing 0.25% trypsin and 0.1% EDTA (Mediatech) warmed to 37 °C was applied apically and basally to dissociate the monolayers into individual cells. The apical volume of trypsin was then neutralized 5–10 min later by the addition of 3 ml of serum-supplemented DMEM. The cell suspensions were washed by centrifuging and decanting 4–5 times using cold phosphate-buffered saline (PBS) (Invitrogen) to remove any residual trypsin, media, calcein, or BSA.

After the final wash, cell pellets were prepared for flow cytometry analysis by resuspension in 0.5 ml of PBS with propidium iodide (Molecular Probes, Cat. #P-3566) and LinearFlow fluorescent polystyrene microspheres (Molecular Probes, Cat. #L-14821) added to make a final concentration of 10 μg/ml and 120,000 microspheres/ml, respectively. Propidium iodide, which stains the nuclei of nonviable cells red, and the fluorescent microspheres, which serve as an internal volumetric standard, were used together to determine the fraction of cells remaining intact and viable after electroporation, as described below.

2.6. Fluorescence analysis

2.6.1. Flow cytometry

Cell viability and molecular uptake of calcein and BSA were quantified using flow cytometry. Data were collected using a FACSort or FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lake, NJ). Each sample was run until data from ~20,000 viable cells were collected by the cytometer. The cytometer was able to distinguish between cells, microspheres, and cellular debris based on size and shape using light scatter measurements (forward scatter and side scatter). The samples were excited using a 15-mW, 488-nm argon laser (Cyonics, now part of Uniphase Corp., San Jose, CA) to analyze for propidium iodide and calcein/BSA fluorescence using a 650 nm longpass filter and 530/30 nm bandpass filter, respectively.

The concentration of viable cells in each sample was determined by multiplying the ratio of viable (propidium iodide negative) cells to microspheres by the known microsphere concentration. Percent viability could then be calculated by normalizing the cell concentration of each electroporated sample to that of the control samples. Calculating viability in this way takes into account cells that may have been physically destroyed during electroporation, as well as those remaining intact, but nonviable (Prausnitz et al., 1993). The average calcein or BSA fluorescence intensity of each sample was converted into the average number of molecules taken up by each viable cell using a series of four quantitative calibration bead standards (Flow Cytometry Standards Corp., Fishers, IN, Cat. #825) with varying fluorescence intensities that corresponded to a known number of fluorescein molecules, as described previously (Prausnitz et al., 1993).

Briefly, a solution containing some amount of the four calibration beads was analyzed at the end of each flow cytometry experiment. Depending on the flow cytometry settings, the four bead populations had certain intensity values that showed a linear relationship with the number of fluorescein molecules associated with each population. The average fluorescence intensities of the analyzed cell samples could then be converted to the average number of fluorescein molecules

based on that linear relationship. Since the intensity of fluorescein is slightly different from that of calcein and BSA, a correction factor was employed to convert from the number of fluorescein molecules to the number of calcein or BSA molecules. The correction factor, which was calculated by fluorimetric analysis of equivalent concentrations of fluorescein and calcein or BSA, was determined to be 1.01 ± 0.20 for calcein and 0.93 ± 0.14 for BSA. Flow cytometry results were analyzed using the WinMDI Flow Cytometry Application (Scripps Research Institute Flow Cytometry Core Facility, http://facs.scripps.edu/).

2.6.2. Confocal microscopy

For analysis by confocal microscopy after electroporation, monolayers were washed as described above and fixed in 3.7% paraformaldehyde solution at room temperature for 20–30 min. Monolayers on membrane supports were then excised and mounted onto microscope slides. A laser scanning confocal microscope (LSM 510, Carl Zeiss Inc., Thornwood, NY) equipped with an argon laser (488 nm) was used to visualize incorporation and localization of calcein and BSA in the monolayers as well as propidium iodide staining of dead cells.

Calculation of intracellular concentration was facilitated by using confocal micrographs to measure cell and nuclear volumes. Based on measurements of 10 cells and 10 nuclei chosen randomly, cell volumes were estimated as $2500 \pm 400\,\mu m^3$ and $1600 \pm 200\,\mu m^3$ for Caco-2 and T84 cells, respectively, and nuclear volumes as $2000 \pm 1000\,\mu m^3$ and $760 \pm 200\,\mu m^3$ for Caco-2 and T84 cells, respectively. The relative size of the nuclei and the cells are consistent with images reported by others (Madara et al., 1987; Lu et al., 1996).

2.7. Long-term monolayer recovery

The recovery of monolayers after electroporation was measured using transepithelial resistance as an indicator of monolayer integrity. Each monolayer was electroporated under sterile conditions in HEPES-buffered DMEM with electroporation pulse conditions of different strengths: “mild”, “moderate”, and “strong”. Although we believe flow cytometry measurements underestimate the actual cell viability in this study, as explained in Section 4, we used approximately 90, 70, and 50% viability according to flow cytometry as indicators of mild, moderate, and strong electroporation conditions, respectively. For Caco-2 monolayers, the conditions that were used to give these viabilities were 50 V–1 ms, 50 V–10 ms, and 100 V–10 ms, respectively. The conditions used for the T84 monolayers were 50 V–5 ms, 50 V–20 ms, and 200 V–5 ms, respectively (see Section 3). Using the Millicell ERS, transepithelial resistance measurements were made immediately (<25 s) and every minute after the electroporation pulse for 5 min. Subsequent measurements were made at 10, 15, and 30 min, 1, 2, 4, 6, 12, and 24 h, and then each day for up to 14 days. Media was replaced every 48 h.

2.8. Statistical analysis

For all of the graphs presented in this study, each data point represents the average (mean) of at least three replicates. The standard error of the mean (S.E.M.) was calculated and used to make the error bars. When a comparison between two or more means was required, a one-way analysis of variance with a 95% level of confidence (ANOVA, $\alpha = 0.05$) was used. Unless otherwise stated, a P-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Electroporation of model intestinal epithelium

To test our hypothesis that polarized model intestinal epithelial monolayers can be uniformly electroporated, we subjected confluent monolayers of Caco-2 and T84 human colon carcinoma cells to single or multiple electric pulses of different voltages and pulse lengths while bathed in an apical solution containing either calcein, a small molecule (623 Da), or fluorescein-labeled bovine serum albumin, a globular protein (BSA; 66,000 Da). In the first part of the analysis, we used confocal microscopy to image the extent and distribution of intracellular uptake of fluorescent molecules by monolayers exposed to electric pulses expected to cause electroporation.

Fig. 2 shows representative confocal micrographs of control and electroporated monolayers that were incubated with calcein or BSA. Control monolayers

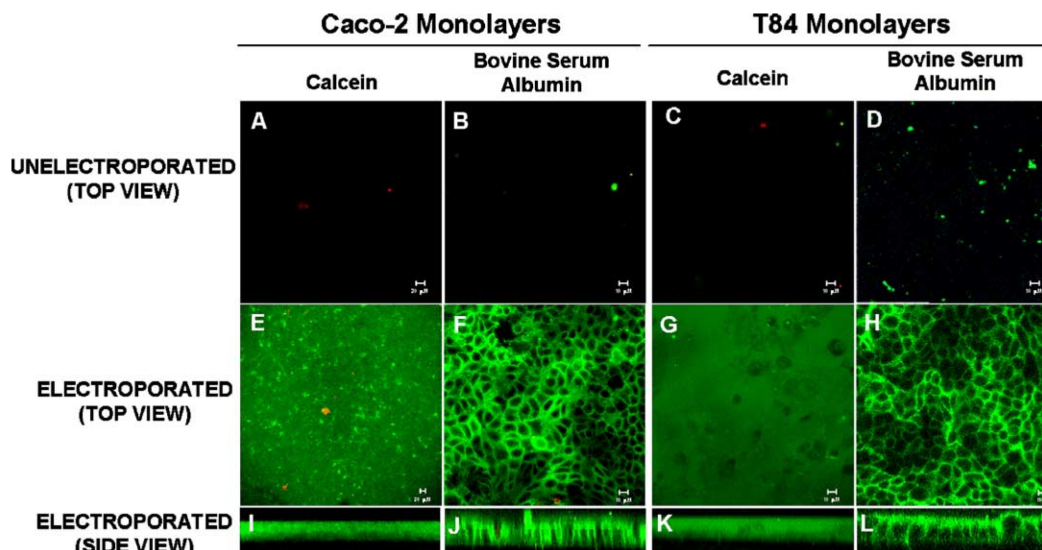

Fig. 2. Confocal images illustrating uptake of calcein and BSA in electroporated Caco-2 and T84 monolayers. While unelectroporated monolayers show almost no fluorescence (A–D), uptake of calcein (E, G) and BSA (F, H) is seen in x–y sections of the monolayers for both cell types. Cross-sectional (z-section) views further demonstrate intracellular localization of the molecules (I–L). Monolayers electroporated with BSA exhibited nuclear exclusion of the protein because of its large size.

exposed to calcein or BSA in the absence of an electric pulse took up essentially no marker compounds (Fig. 2A–D), whereas monolayers exposed to a single electroporation pulse contain large amounts of intracellular calcein (Fig. 2E and G) and BSA (Fig. 2F and H). Calcein delivery appears to occur throughout each cell, whereas BSA is present throughout the cytosol, but excluded from the nucleus. This exclusion is probably due to the large molecular size of BSA, which limits its diffusion through the nuclear pore complex (Talcott and Moore, 1999). The lack of fluorescence in the nucleus also suggests that there was probably little or no degradation of BSA by proteolytic enzymes. Small, fluorescein-labeled protein fragments would have passed easily through the nuclear pore complex, which has a molecular weight cutoff of ∼50 kDa for passive diffusion.

These observations are further supported by images from cross-sectional views of monolayers, which show calcein throughout the cell interiors (Fig. 2I and K) and BSA filling the cytoplasm outlining the nuclei (Fig. 2J and L). In addition, when monolayers were observed under low power magnification, the distribution of calcein and BSA uptake appeared to occur uniformly over the entire monolayer, i.e. there were no 'hot spots' of high fluorescence intensity (data not shown).

3.2. *Quantification of molecular uptake and cell viability*

3.2.1. *Effect of pulse voltage and length*

Having established that cells forming a functional epithelial monolayer can be uniformly electroporated, we next used flow cytometry to quantify the average number of calcein and BSA molecules taken up by each cell and the loss of cell viability associated with electroporation as a function of pulse voltage and length. This analysis showed that it is possible to transport more than 10^6 calcein molecules per cell and more than 10^5 BSA molecules per cell into both Caco-2 and T84 monolayers using electroporation (Fig. 3A–D). Moreover, these average uptake values represent a homogeneous response of the cells in the monolayer, as evidenced by the approximately Gaussian distribution about the mean uptake level among the 20,000 viable cells per sample analyzed by flow cytometry (data not shown). This uniform response is consistent with previous observations for electroporation of cells in suspension (Prausnitz et al., 1993; Canatella et al., 2001).

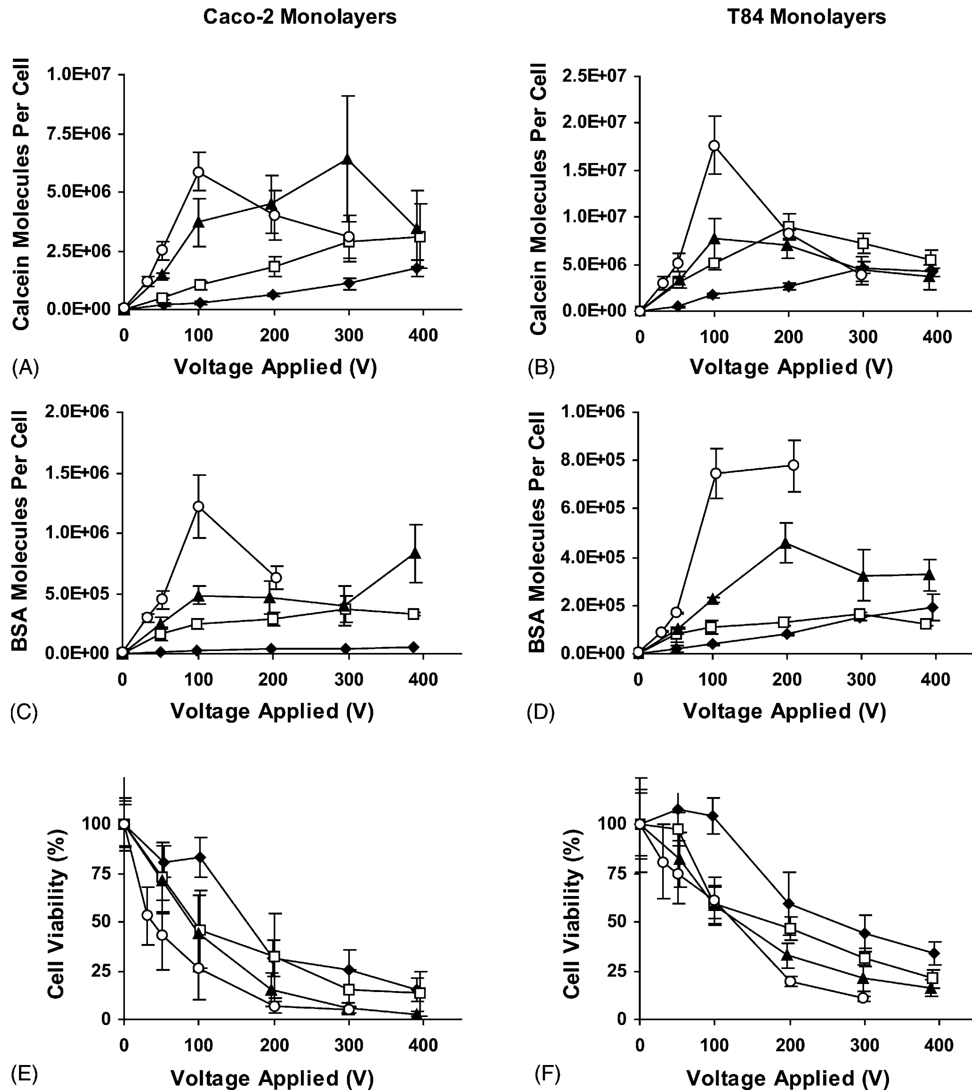

Fig. 3. Dependence of intracellular uptake and cell viability on electroporation voltage and pulse length. Uptake of calcein and BSA by epithelial monolayers increased as voltage and pulse length were increased (A–D). Cell viability decreased with voltage and pulse length (E, F). Pulse lengths were 1 ms (◆), 5 ms (□), 10 ms (▲), and 20 ms (○).

Fig. 3 also shows that the average number of calcein and BSA molecules taken up by each Caco-2 and T84 cell generally increased with increasing applied voltage to an apparent plateau (one-way ANOVA, $P < 0.05$). Uptake also increased with increasing pulse length, which was statistically significant for voltages less than 300 V ($P < 0.05$).

The number of molecules delivered per cell should be thermodynamically limited to an intracellular concentration equal to the extracellular concentration, which was 100 μM for calcein and 10 μM for BSA. Based on measurements of cell and nuclear volumes (see Section 2), thermodynamic equilibrium is expected for calcein at 1.5×10^8 molecules per Caco-2 cell and 9.4×10^7 molecules per T84 cell and for BSA, assuming nuclear exclusion, at 3.3×10^6 molecules per Caco-2 cell and 4.9×10^6 molecules per T84 cell. Fig. 3 indicates that although large num-

bers of molecules were delivered, uptake of calcein and BSA were usually 1–2 orders of magnitude below equilibrium, which is also consistent with previous measurements of cells electroporated in suspension (Canatella et al., 2001).

The above analysis discussed the number of molecules delivered into those cells that remained viable after electroporation. However, electroporation can render cells non-viable. Fig. 3E and F show that the viability of electroporated cells generally decreased with voltage ($P < 0.05$) and, to a lesser extent, with pulse length. This observation indicates that there is a tradeoff between conditions that yield large levels of uptake (i.e. long pulses and possibly high voltages) and those that maintain high viability (short pulses and low voltages). The significance of this sometimes large viability loss and its possible overestimation by flow cytometry is discussed further below.

3.2.2. *Effect of pulse number*

We performed additional experiments to determine the effect of multiple pulses on calcein uptake and cell viability. Between 1 and 20 pulses were applied over a range of pulse lengths, while the applied pulse voltage was held constant at 50 V. Fig. 4A and B show that as the number of pulses was increased and as pulse length was increased, uptake increased in both Caco-2 and T84 monolayers ($P < 0.05$). In Fig. 4C and D, cell viability generally decreased as the number of pulses and pulse length were increased (although not always with statistical significance; $0.000 < P < 0.78$).

Since increasing the number of pulses and increasing pulse length have similar effects (i.e. they both increase the duration of exposure to electroporation), we wanted to determine whether single long pulses yielded the same effects as several short pulses having the same total exposure time (TET; defined as the product of pulse length and number of pulses). Using combinations of pulses having TET of 5, 10, and 20 ms, we found that uptake of calcein by both Caco-2 and T84 cells was not statistically different at constant TET (Fig. 5A and B; $P > 0.05$). Similarly, cell viability in both types of monolayers was statistically

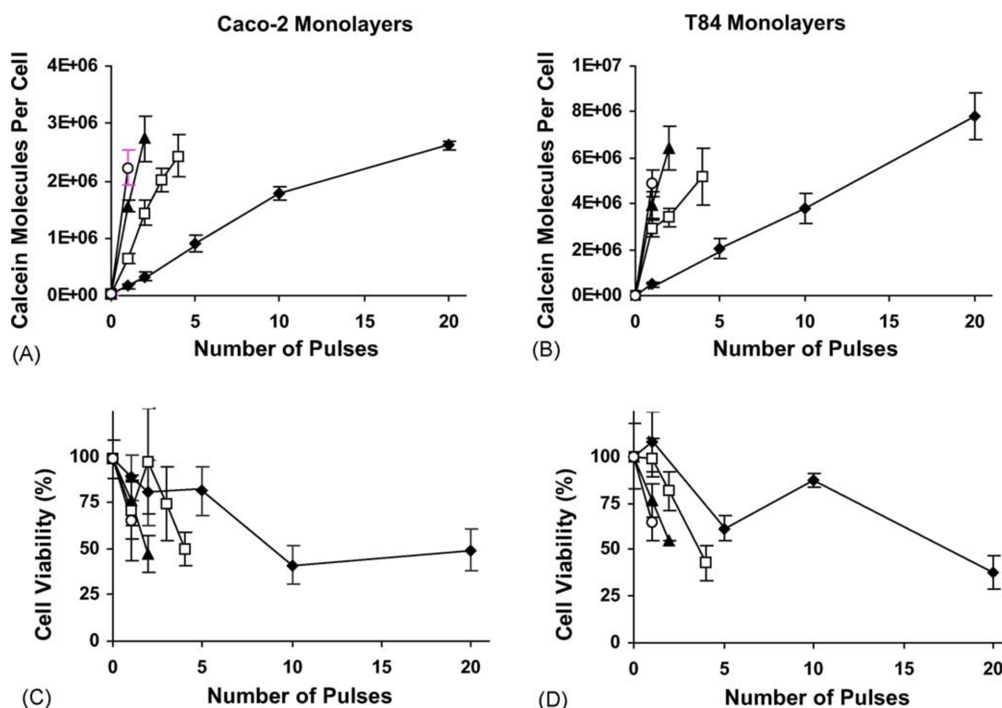

Fig. 4. Dependence of intracellular uptake and cell viability on electroporation pulse number and pulse length. Calcein uptake increased with increasing pulse number and pulse length (A, B). Cell viability decreased with increasing pulse number and pulse length (C, D). Applied voltage was held constant at 50 V. Pulse lengths were 1 ms (◆), 5 ms (□), 10 ms (▲), and 20 ms (○).

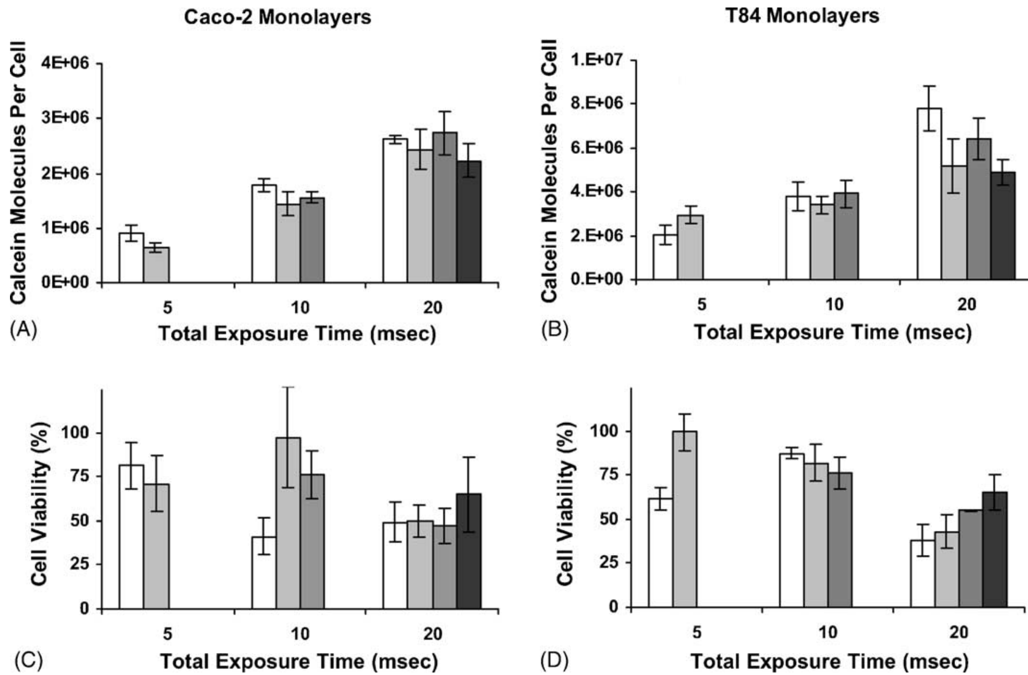

Fig. 5. Intracellular uptake of calcein (A, B) and cell viability (C, D) were similar for the same total exposure time to electroporation (TET; defined as the product of pulse length and number of pulses). Data are replotted from Fig. 5. Pulse lengths were 1 ms (□), 5 ms (▨), 10 ms (▨), and 20 ms (■).

indistinguishable at the same TET (Fig. 5C and D; $P > 0.05$).

3.3. Long-term monolayer recovery

The second hypothesis for this study proposed that loss of viability and tissue barrier function caused by electroporation can recover rapidly. Using flow cytometry, we measured decreases in cell viability following electroporation. Since epithelial monolayers are comprised of proliferating cells that can repair possible damage caused by electroporation, the recovery kinetics of monolayers after electroporation were monitored to determine which conditions permitted rapid recovery. Transepithelial resistance (TEER) was used as an indicator of monolayer integrity.

Fig. 6A and C show the long-term recovery of Caco-2 monolayer resistance after electroporation at a “mild”, “medium”, or “strong” condition (see Section 3.3). There was an initial drop in TEER immediately after each pulse, which then recovered at varying rates depending upon the condition applied (Fig. 6A). Mildly electroporated monolayers (uptake $\sim 2 \times 10^5$ calcein molecules per cell, mpc, viability ~81%; see Fig. 3) were able to recover their original resistance in less than 6 h. Moderately electroporated monolayers (uptake $\sim 2 \times 10^6$ mpc, viability ~71%) recovered within a day. Finally, strongly electroporated monolayers (uptake $\sim 4 \times 10^6$ mpc, viability ~44%) did not recover their initial resistance until more than a week later.

Fig. 6B and D show the recovery of electroporated T84 monolayers, which behaved similarly to the Caco-2 monolayers. For mild electroporation (uptake $\sim 3 \times 10^6$ calcein mpc, viability ~98%), T84 monolayers recovered their initial resistance in 24 h. Monolayers that were moderately electroporated (uptake $\sim 5 \times 10^6$ mpc, viability ~75%) recovered within a few days, and those that were strongly electroporated (uptake $\sim 9 \times 10^6$ mpc, viability ~47%) required more than a week to recover. T84 recovery rates were slower than Caco-2 rates probably because of their

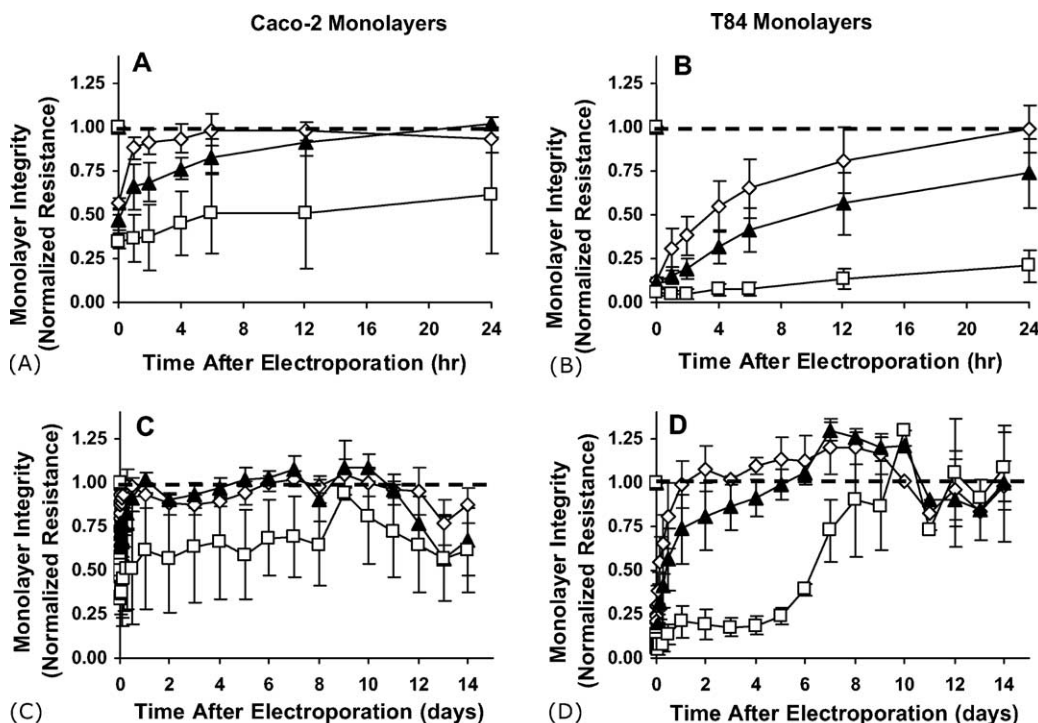

Fig. 6. Kinetics to restore barrier integrity of monolayers in the first 24 h (A, B) and 14 days (C, D) after electroporation. Caco-2 (A, C) and T84 (B, D) monolayers were electroporated under mild (◇), moderate (▲), and strong (□) electroporation conditions (see Section 3.3). Monolayer integrity was measured using transepithelial electrical resistance (TEER) of the monolayers, which was normalized relative to unelectroporated control monolayers and initial resistance.

longer doubling time, i.e. 60 h versus 30 h for Caco-2 cells (Dharmsathaphorn and Madara, 1990; Gres et al., 1998). Together, these recovery experiments show that on the order of 10^6 molecules per cell can be delivered under mild to moderate electroporation conditions into monolayers that require hours up to one day to fully recover barrier integrity, which is an indicator of good tissue function and health.

4. Discussion

In this study, Caco-2 and T84 epithelial monolayers were electroporated using a range of voltages, pulse lengths, and pulse numbers. Quantification of molecular transport and cell viability showed that for both cell types, uptake of calcein and BSA increased with increasing voltage and pulse length, reaching levels up to 10^7 molecules per cell. Monolayer recovery experiments showed that even under relatively mild electroporation conditions (∼50 V), monolayers were able to take up many thousands to millions of molecules per cell and still recover barrier properties within hours. This ability to take up molecules and then recuperate quickly should be useful for laboratory studies of gastrointestinal inflammation or other intestinal conditions. For example, one could electroporate an experimental drug or protein into an epithelial monolayer, wait 1–2 days and then carry out tests to evaluate the resulting effects on monolayer function.

There are also potential clinical applications for electroporation of epithelia, e.g. treatment of intestinal disorders by introduction of drugs, proteins, or genes. Although electroporation causes some temporary cell damage, it still might be useful clinically as long as the epithelium is able to recover and regain confluence relatively quickly. When superficial wounds to the epithelial lining of the intestinal wall naturally oc-

cur in the body due to physical injury, microorganisms, or other agents, cells adjacent to the wound quickly dedifferentiate and migrate to cover the exposed area (Nusrat et al., 1992; Dignass, 2001). This frequent and natural healing process, called restitution, occurs within minutes to hours and serves to maintain the barrier necessary to protect the body from the external environment. The electroporation conditions from which monolayers recovered in less than 24 h in this study may similarly permit rapid resealing of epithelia in vivo. Consequently, if one were to apply electroporation in vivo to treat an intestinal disorder, it may be acceptable to kill, for example, 10% of the cells in a small treatment area, since the intestinal epithelium is able to repair itself quickly. Additional studies are needed to test this hypothesis and evaluate possible uses of electroporation in vivo.

Although we have provided measures of cell viability from flow cytometry experiments (e.g. Figs. 3–5), we believe these may overestimate the loss of cell viability. For example, confocal images of intact electroporated monolayers show far fewer dead cells (by propidium iodide staining) than what is observed by flow cytometry analysis of cells dissociated from similarly treated monolayers (images not shown). Moreover, monolayer resistance measurements made just after electroporation at mild to moderate conditions show drops to at most 50% (Caco-2) or 20% (T84) of pre-electroporation values. These levels of resistance drops have been shown to occur when tight junctions between cells are disrupted (Liu et al., 2000; Ma et al., 2000), and do not require killing of large numbers of cells. If up to 30% of the cells in the monolayer were destroyed, as flow cytometry measurements under the same conditions estimated, then based on cell doubling times (see Section 3), the process of replicating new cells to regain confluence should have required more than the 24 h we observed.

For these reasons, we believe that cell viability losses calculated using flow cytometry are overestimated probably due to monolayer dissociation and artifacts of preparing cells for flow cytometry analysis. Although viabilities were determined by comparing to unelectroporated controls, cells that have been electroporated could be more fragile and, thus, more likely to be adversely affected by the trypsin and physical treatments used to dissociate the monolayers. Since it appears that the cells are better able to recover when left in monolayer form, transepithelial resistance may be a more useful indicator of monolayer viability than flow cytometry measurements.

Multiple pulse experiments indicated that the total 'on time' of a pulse determined levels of uptake and viability, independent of whether that 'on time' was achieved through a single long pulse or multiple shorter pulses (Fig. 5). In contrast, results from multiple pulse experiments conducted with prostate cancer cells in suspension reported by Canatella et al. (2001) showed more uptake and lower cell viability when single long pulses were applied than when several short pulses were applied. This difference may be due to differences in cell type, suspension versus monolayer configuration, or error bars that may obscure small variations in the measurements.

The choice of an optimal electroporation condition will depend on the application for which it is being used. Most applications will require efficient delivery of a molecule of interest while minimizing cell death. Typically, the goal would be to alter or restore the functional processes of living cells. In such cases, short, low voltage pulses (e.g. 50 V, 1–10 ms) should be most effective. If cell death is not a concern, and more extensive uptake by the surviving fraction of cells is needed, then much stronger electroporation conditions could be used.

In conclusion, this study provided data in support of the hypotheses that (1) electroporation can deliver small molecules and macromolecules in a manner that is uniform in both spatial distribution and intracellular concentration, and (2) loss of viability and tissue barrier function caused by electroporation can recover rapidly. Electroporation of polarized intestinal epithelial monolayers was demonstrated for the first time, showing that intestinal epithelial monolayers can be electroporated to induce extensive, uniform uptake of extracellular, membrane-impermeant molecules, including proteins. Temporary loss in monolayer integrity was a side effect of electroporation, but could be repaired within a day. The results of these experiments suggest the feasibility of using electroporation to deliver drugs, proteins, and other therapeutic molecules into intestinal and, possibly, other epithelia for development of improved laboratory models of intestinal epithelium, as well as possible local drug and gene therapy of intestinal disorders.

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