Methods for *in Vivo* Tissue Electroporation Using Surface Electrodes

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Electroporation of tissues has many potential biomedical applications, including transdermal and targeted drug delivery. Although there are established protocols for electroporation of single cells, electroporation of tissues remains largely unexplored. Here we describe methods for *in vivo* tissue electroporation with surface electrodes, where either (a) molecules are provided at the outer surface of skin and electroporation is caused in order to transport molecules across the skin or (b) molecules are injected into an animal and internal tissue cells are electroporated to enhance uptake of the injected molecules. Factors considered in our experimental design include selection of electrode material and electrode/tissue geometry. Electrode materials were used which could accommodate a high instantaneous current density and were expected not to form harmful electrochemical products. Due to the complex heterogeneous nature of tissues, the choice of electrical pulse parameters was guided by, but not based solely on, results from single-cell systems. Multiple pulses appeared to be useful in causing electroporation of tissues located deeper than the skin's stratum corneum. Secondary problems which resulted from the direct muscle stimulation associated with pulsing were also considered. Developing methods for *in vivo* tissue electroporation required an interdisciplinary approach, involving both high-voltage electric fields and the basic properties of living tissue. © 1993 Academic Press, Inc.

Electroporation involves the creation of a transient high-permeability state (electropermeabilization) in lipid bilayer membranes by the application of an electric field pulse (1–5). This increased permeability is often reversible and is believed to be due to the formation of short-lived aqueous pores within the membrane. Causing electroporation of cell membranes is a well-established technique, finding extensive application as a method of introducing exogenous genetic material into cells, often thereby transfecting them (1–5). However, electroporation does not take place only in cells, but appears to be universal, as it occurs in lipid bilayer membranes, largely independent of their composition or structure. For example, electroporation has been demonstrated in many different mammalian, plant, yeast, and bacterial cells, as well as in artificial planar and spherical membranes (1–5). More recently, electroporation of cell membranes and other lipid-containing structures within tissues has been demonstrated (6–21).

The prospect of transiently enhancing transport across a tissue by electroporation suggests a variety of compelling research and biomedical applications (1–5). For example, electroporation of cells within a selected tissue could cause increased local uptake of drug, allowing drug targeting. Moreover, some drugs are ineffective because they are unable to cross cell membranes under normal physiological conditions. However, electroporation could allow these drugs to enter their target cells and become therapeutically useful. Another possible application is electroporation of skin to enhance transdermal drug delivery.

These potential applications motivate our study of
tissue electroporation. However, the heterogeneous composition and complex geometry of tissues and the use of living animal models add new complexities not present in isolated-cell electroporation. For example: (a) concern for animal health, possible pain, and nervous stimulation is required; (b) the use of multiple pulses increases the need to understand and control electrode properties and electrochemistry; and (c) heterogeneous and changing tissue electrical properties suggest the need for new pulse protocols and make interpreting experimental results more challenging. This paper describes how we have addressed these methodological issues for our in vivo tissue electroporation studies.

To date, electroporation using living animals has been reported by only a few research groups. Okino et al., first demonstrated in rats that electroporation at the site of a tumor could increase the effect of a chemotherapeutic agent on tumor destruction relative to pulse-only or drug-only controls (6–8). Following systemic administration of bleomycin, cylindrical electrodes were inserted through the skin on opposite sides of the tumor (2- to 2.5-cm spacing) and a single square-wave or exponential-decay pulse of ≤10 kV and ≤7.25 ms was applied. These electrodes produce a highly non-uniform electric field, with a very large field near the electrodes.

Related studies have been done in mice by Mir et al., employing surface, parallel-plane electrodes placed across the tumor (6.6-mm spacing) (9–12). These electrodes provide a nominally uniform electric field, with departures from uniformity due mainly to tissue heterogeneity. A series of 8 square-wave pulses (100 μs width) at 1 pulse per second (pps) was applied using 900–1500 V/cm. Mir and co-workers have also shown tumor regression in seven human patients by administering bleomycin with a similar pulsing protocol (11). Both Okino's and Mir's groups report transient edema as the only significant side effect. Finally, using a different protocol, Mir and co-workers have recently demonstrated electroporation-enhanced cancer chemotherapy for treatment of brain tumors in rats (13).

Of relevance to gene therapy, Titomirov et al. have used surface electrodes to electroporate dermal cells in mice, thereby enhancing uptake and resulting in expression of plasmid DNA injected subcutaneously (14). Two exponential-decay pulses of opposite polarity were applied at 400–600 V, 100–300 μs, resulting in tissue necrosis at higher voltages.

Grasso et al. have demonstrated electrofusion of cells to rabbit corneal epithelium, potentially important for novel approaches to drug delivery, wound healing, and development of new animal models (15–17). Electrofusion is a phenomenon which is believed to be mechanistically related to electroporation (1–3). Using an electrode which fit the curvature of the eye and a counter electrode attached to the buccal mucosa, three pulses of 20 V, 20 μs were applied at 1 pps. Ocular inflammation or more severe damage was not observed.

Work from our group has emphasized electroporation of skin, which we believe involves creation of aqueous pathways within the multilamellar intracellular lipids of the stratum corneum, skin's outer and least permeable layer (19–21). Possible applications include enhanced transdermal drug delivery and non-invasive sensing of interstitial fluid components. We have demonstrated up to 10,000-fold increases in transdermal flux which are largely or completely reversible in vitro; similar fluxes have been measured in vivo. In separate but related studies, we have also started work in the area of locally enhanced drug delivery by in vivo electroporation of tissues for treatment of leishmaniasis and other exploratory studies.

MATERIALS AND METHODS

Transdermal Delivery

Unlike the other applications described above, electroporation of skin for transdermal drug delivery requires that a solution or gel containing drug be present between the electrode and the skin. Figure 1 shows the apparatus we developed for this purpose, based in part on conventional in vivo transdermal drug delivery techniques and in part on the approach taken by Titomirov et al. (14).

CD hairless rats (Charles River Laboratories, Wilmington, MA) were anesthetized with 75 mg/kg ketamine HCl (Ketaset, Aveco Co., Ft. Dodge, IA) and 10 mg/kg xylazine (Rompun, Mobay Corp., Shawnee, KS) by intraperitoneal injection. Animal care was in accordance with institutional guidelines. Depth of anesthesia was assessed using corneal and pedal reflexes. Additional 1/3 doses were given to maintain sedation (every 30–45 min). Lubricant ointment (Artificial Tears, Vedco, St. Joseph, MO) was placed on the eyes to prevent drying.

As shown in Fig. 1, two glass bells (~4 ml, 2.8 cm²), fitted with rubber septa and Ag/AgCl electrodes (4-mm-diameter disks, ~1 cm from skin, In Vivo Metrics, Healdsburg, CA), were affixed with cyanoacrylate adhesive (Permabond, Englewood, NJ) to pinched skin from the caudodorsal surface of the rat. Excess adhesive was added outside the bells to ensure a good seal. The area where the bells contacted the skin was then covered with silicone lubricant (Dow Corning, Midland, MI) to ensure electrical insulation. The bells and pinched skin were held in place with a nonconductive pinch clamp. One bell was then filled with isotonic phosphate-buffered saline (PBS, pH 7.4), and the other with a saturated calcein solution in PBS. Calcein (Molecular Probes, Eugene, OR) is a fluorescent model for a moderate-size polar drug. Solutions were injected through each septum with a needle and syringe; dur-
FIG. 1. Apparatus for transdermal drug delivery by skin electroporation. (A) An anesthetized hairless rat with the apparatus attached to pinched skin. (B) A close-up of the apparatus. (C) A schematic of the apparatus showing two glass bells, filled with saline solutions, adhered to a rat. On each, an electrode was inserted through a septum. Adhesive and a coating of silicone lubricant along the edge of the bell provided electrical insulation and prevented leakage. A nonconductive clamp held the apparatus in place (not shown).

An electric pulse (exponential-decay constant, $\tau = 1.2 \text{ ms}$) was then applied once every 5 s for 1 h, a much longer electrical exposure than in previous electroporation studies. Transtissue pulse voltages between 75 and 300 V were investigated. Rats were monitored visually throughout. The experiment was briefly suspended when administration of anesthetics was required to maintain deep sedation. After the end of 1 h of pulsing, the bells were drained, again using an extra needle to prevent pressure changes, and carefully removed using Q-tips dipped in acetone to dissolve the adhesive. Some skin, probably the stratum corneum, generally remained adhered to the bells, leaving a thin ring of exposed pink tissue.

At a few time points between 15 and 120 min after pulsing, blood samples (~0.5 ml) were taken from the lateral tail vein with a preheparinized 25-gauge butterfly needle and transferred into a serum separator tube (Microtainer, Becton–Dickinson, Rutherford, NJ). The tube was spun at 1000g for 5 min. The plasma was then collected for analysis by spectrofluorimetry (Fluorolog-2, Model F112AI, SPEX Industries, Edison, NJ). Samples were excited at 488 nm and emission spectra were obtained between 505 and 535 nm, as shown in Fig. 2. Background signal was subtracted, as determined with control plasma samples. Fluorescence was calibrated against known concentration standards.

The appropriate volume of distribution of calcein within the rat was determined by measuring plasma
concentrations over time following intravenous and subcutaneous injections of known amounts of calcein. Maximum plasma concentrations were measured 30–60 min after injection, suggesting that significant metabolism or elimination of calcein did not occur over that period (22,23). The volume of distribution determined from these measurements was 20% of total rat volume (24), which is equal to the volume of the extracellular aqueous compartment (25). Given the very hydrophilic nature of calcein (26), distribution throughout all extracellular aqueous regions is a reasonable assumption.

Rats were kept comfortably warm and under observation until they recovered from the anesthesia. They were then checked at least once daily to assess any adverse effects from the pulsing procedure. Mild, transient erythema and edema were generally seen over the area of electrical contact with the skin (i.e., where the solutions within the bell chambers touched the rat) immediately after pulsing for transdermal voltages below 150 V; more pronounced erythema and edema were observed at higher voltages. Deep tissue necrosis, evident from macroscopic examination, was observed in one rat 2 days after pulsing at the highest voltage used (300 V). No other rats responded to gentle prodding or protected the sites of pulsing, suggesting that they were not painful and that severe damage had not occurred.

**Locally Enhanced Delivery**

Unlike the transdermal procedures, protocols used by us for locally enhanced drug delivery were adapted from Mir et al. (9,10). Parallel-plane stainless steel electrodes, with a layer of conductive gel (Signa Gel, Parker Laboratories, Orange, NJ or Redux Paste, Hewlett-Packard, Waltham, MA) coating the surfaces, were applied to a mouse's skin shortly after local or systemic injection of drug. Two electrode designs were employed: one with a fixed interelectrode spacing and one with an adjustable interelectrode spacing.

The fixed-spacing design (Fig. 3A) was used in studies involving leishmaniasis, since the lesions being treated did not vary much in size. The electrodes were placed across the lesion at the base of the tail and held firmly in place. For other studies, the adjustable-spacing design (Fig. 3B) was employed to accommodate pulsing sites of different sizes. Electrodes were mounted on a nonconductive, plastic micrometer (Manostat 15-100-100, Fisher Scientific, Pittsburgh, PA) so that the interelectrode spacing could be easily determined. In this way the nominal applied electric field could be calculated for a given pulse voltage.

**RESULTS AND DISCUSSION**

**Transdermal Delivery**

We have obtained data which suggest that electro­poration of rat skin in vivo occurs and results in large concentrations in rat plasma collected 15–120 min after electroporating rat skin: (a) pulsed at 300 V, (b) pulsed at 75 V, (c) unpulsed control, (d) normal rat plasma (see text for pulsing protocols). Samples were excited at 488 nm; emission spectra are shown, with maxima between 510 and 515 nm. After calibration against known standards, absolute calcein plasma concentration was determined.

![FIG. 2. Spectrofluorimetric analysis showing calcein fluorescence in rat plasma collected 15–120 min after electroporating rat skin: (a) pulsed at 300 V, (b) pulsed at 75 V, (c) unpulsed control, (d) normal rat plasma.](image)

**Locally Enhanced Delivery**

![FIG. 3. Design of electrodes used for tissue electroporation. (A) Side view of parallel-plane electrodes with fixed interelectrode spacing. This electrode design was used in leishmaniasis studies, where lesions being treated did not vary much in size. (B) Top view of parallel-plane electrodes with adjustable interelectrode spacing. Mounting the electrodes on calipers allowed determination of interelectrode spacing. This electrode design was used to accommodate treatment of pulsing sites of different sizes. For clarity, wires and conductive paste are not shown.](image)
Electrode Material

We used homogeneously mixed Ag/AgCl electrodes for the transdermal delivery studies, primarily for two reasons. First, these electrodes were able to pass the large instantaneous current densities associated with the high-voltage pulses applied. Using a current sampling resistor, we have measured peak instantaneous current densities up to 1 A/cm². In contrast, Ag wire electrodes electrochemically plated with AgCl were problematic, as the outer, insulating layer of AgCl can detach during pulsing. Electrodes made of a homogenous mixture of Ag and AgCl did not have this problem.

Second, Ag/AgCl electrodes minimized harmful electrochemical effects, such as pH changes due to hydrolysis, which are known to cause skin irritation (27). This is particularly important for transdermal delivery applications, where many pulses might be given over long periods of time. However, even the Ag/AgCl electrodes used appeared to hydrolyze water at the high current densities used, indicated by the formation of gas bubbles. Although pH changes were not observed in our buffered solutions, the formation of gas elevated the pressure within the bell chambers; occasional insertion of a needle through the septum alleviated the problem by returning the pressure to atmospheric.

Electrode Position

Most investigators have placed electrodes at a single location and applied one or more pulses. However, we found that it may be advantageous to move the electrodes to different locations near the site being electroporated. Given the heterogeneities of tissue and the asymmetric shapes of cells, a pulse in one location may not electroporate a given cell, while a shift in field orientation may subsequently result in electroporation. However, when electrodes were moved, the skin had to be cleansed of any residual conductive paste to avoid electrical shorting. Also, to minimize cardiac risk, we placed electrodes away from the heart.

Electrical Parameter Selection

Mir et al. have based their selection of electrical parameters largely on work in vitro, coupled with further optimization in vivo (9,10), while Okino et al. have studied pulse settings in vivo (6–8). Even for simpler in vitro systems, such as cells in suspension, choosing electrical parameters is highly empirical due to incomplete understanding of electroporation. Given the greater complexity of tissues, it is often difficult to translate such empirical in vitro parameters directly to in vivo applications. Nevertheless, in vitro results provide useful guides for optimization in vivo.

With this in mind, experimental results from single-cell electroporation in part motivated our work on locally enhanced delivery to treat leishmaniasis. It is known that larger cells will electroporate at lower field strengths than smaller cells (1–5). This should cause selective electroporation of macrophages, which are larger than most other cells (28). Because leishmania are found predominantly in macrophages within the skin (29), drug delivery could be targeted to leishmania in macrophages by selection of appropriate electroporation protocols.

Determination of appropriate electrical parameters, however, was also dictated by concepts not evident from single-cell electroporation. When using surface electrodes to electroporate cells deeper than the skin’s outer layer, the stratum corneum, we chose to use multiple pulses. Since the stratum corneum normally has a resistance much greater than that of deeper tissues, the electric field concentrates at the stratum corneum. As a result, deeper cells experience much weaker fields, perhaps insufficient to electroporate. However, the stratum corneum can also be electroporated, dramatically reducing its resistance after just one pulse, where significantly lowered resistance can persist for seconds to hours (19–21). Therefore, we used multiple pulses, whereby an initial pulse was given to electroporate the stratum corneum, making subsequent pulses yield greater internal electric fields, more likely to electroporate deeper cells.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Pulse voltage (V)</th>
<th>Transdermal calcein flux (nmol/cm² h)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>75</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>150</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
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*Note. A single exponential-decay pulse with time constant $\tau = 1$ ms was applied every 5 s for 1 h, following the protocol described in the text. Interelectrode spacing was ~3 cm, including pinched skin of ~1 cm thickness (see Fig. 1). The calcein flux detection limit was on the order of 0.1 nmol/cm² h. Each data point represents results from one to two different rats.*
The heterogeneous electrical properties of tissues such as skin point to another difficulty associated with basing tissue protocols on single-cell results. For heterogeneity, and the resultant heterogeneity in field strength (e.g., kV/cm). However, the high resistance of the stratum corneum is an example of how tissue cations. We have found it most useful to record both pulse strengths are uniform within a tissue as an initial approximation.

Finally, we also found it important to verify that the pulser used was capable of delivering square-wave pulses without overloading. During an electric pulse, the apparent resistance of tissue became very small due to electroporation, resulting in large instantaneous current densities. As an example, when we pulsed mouse legs at voltages between 1 and 2 kV (100 μs duration, 8 pulses, 1 pps, 3–10 mm electrode spacing, 1 cm² contact area) to enhance transport locally, the apparent resistance during the pulse was approximately 400 ohms, resulting in currents of a few amperes (unpublished data). Thus, to produce a square-wave pulse of 2 kV for this experiment, the pulser needed a power rating of at least 10,000 W. Because most square-wave generators do not have such high power ratings, we used the Velonex (Santa Clara, CA) Model 350 pulser with a V1743 output plug-in unit, which is capable of delivering 2 kV into a 200-ohm load. Only when an oscilloscope and appropriate sampling circuit were used was it apparent that a square-wave pulser with an insufficient power rating had delivered a pulse of incorrect magnitude or waveform. A consequence of overloading a pulser is that the nominal electric field can be significantly smaller than estimated from the pulser setting and interelectrode electrode spacing.

Side-Effects due to Pulsing

While animals insufficiently anesthetized responded to electroporation pulses, we also observed direct stimulation of motor nerves in fully anesthetized animals. This phenomenon is well known (30). Although the movement caused by such stimulation was not inherently problematic, we encountered two significant difficulties. First, in our transdermal experiments, involving application of multiple pulses to skin over the lower back, the rats' hind legs kicked in response to each pulse. The intensity of kicking varied with the applied voltage and electrode position. This effect lessened significantly within minutes, presumably due to muscle fatigue. However, the sustained kicking apparently caused some damage, evidenced by blood in the urine observed in some of the first rats used. Placement of subsequent rats on a cushioned surface alleviated this problem.

A second concern became apparent when pulsing leishmania lesions at the base of the tail in mice. Again, each pulse caused the mouse's hind leg muscles to contract. In this case, the electrodes (Fig. 3A) were held by the experimenter firmly in contact with the mouse. Immediately after pulsing, a mild abrasion to the skin under the electrodes was often evident. We believe it was due, at least in part, to the animal forcefully jumping into the thin metal electrodes. An apparatus using electrodes attached to the animal might have alleviated this problem, as would have securing the animal more firmly in place.

In other experiments for locally enhanced drug delivery, kicking of the hind legs was eliminated by electrode design and placement. The caliper electrodes (Fig. 3B) were positioned on either side of the targeted site such that they avoided encompassing most muscle masses of the leg. Also, electrodes were not placed near the spine, in contrast with other studies. As a result, this optimized electrode placement eliminated most muscle stimulation. Nevertheless, a slight limp that lasted for several minutes after mice awoke suggested that some muscular or nervous fatigue had occurred. Mild, transient erythema was also observed in the area where pulsing had taken place.

A final complication concerned anesthetized animals being awakened by pulsing. Sometimes animals which appeared to be fully anesthetized and remained so during a few pulses would start to wake up during subsequent pulses. Lack of deep anesthesia was demonstrated by animal vocalization and additional movement which followed the twitch caused directly by the pulse.

Damage to Tissue

At the membrane level, electroporation is a fundamentally gentle phenomenon, known to be reversible over a range of conditions (1–5). However, the electroporation literature makes clear that secondary effects of electroporation are capable of killing cells in suspension, presumably affecting cells in tissues in a similar manner (1–5,31). Moreover, thermal and pH burns may also occur. Nevertheless, our work and that of others suggest that severe tissue necrosis and other macroscopically visible damage due to electroporation do not occur over a useful range of conditions, as described in the Introduction and under Materials and Methods (6–21). However, careful biochemical and pathological studies of the effects of tissue electroporation are needed.
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