Percutaneous Penetration Enhancers

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Chapter 15.4

Electroporation

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CONTENTS

I. INTRODUCTION

Transdermal drug delivery offers many potential advantages over conventional methods of drug administration.[1-4] However, very few drugs can be administered transdermally at therapeutic levels, due to the low permeability of human skin. The remarkable barrier properties of skin are attributed primarily to the stratum corneum (SC), the skin's outer layer. The SC is a dead tissue composed of flattened cells filled with cross-linked keratin and an extracellular matrix made up of lipids arranged largely in bilayers.[5,6] Intercellular pathways are generally believed to be the most important routes for transdermal transport. Therefore, permeabilization of the lipid bilayers occupying these intercellular pathways would be expected to increase transdermal transport.

A number of chemical, electrical, and other approaches to enhance transport across skin have found varied success, as discussed elsewhere in this book. This chapter presents a novel approach to enhancement involving electroporation, an electrical phenomenon known to dramatically and reversibly alter lipid bilayer permeability. We examined the possibility of electroporating the intercellular lipid bilayers of the SC to enhance transdermal drug delivery. In a recent paper[7] we discussed skin electroporation in detail. Here, we provide a review of this topic, as well as more recent developments.

II. ELECTROPORATION OVERVIEW

Electroporation, which includes electropermeabilization, involves the creation of aqueous pathways in lipid bilayer membranes by the application of a brief electric

field pulse.[8–12] Permeability and electrical conductance of lipid bilayers, such as cell membranes, are increased by many orders of magnitude. Moreover, the associated local electric field can contribute to transmembrane molecular transport by electrophoresis and/or electroosmosis. These membrane changes can persist for up to hours, but are reversible or irreversible, depending mainly on pulse magnitude and duration. Electroporation has been demonstrated in many different mammalian, plant, yeast, bacterial, and other cells, as well as in artificial planar and spherical membranes. Thus, electroporation appears to be universal in lipid bilayers, with onset largely independent of their exact composition or structure. Although the creation of transient aqueous pathways, or electropores, is the proposed mechanism by which electroporation occurs, the exact physical nature of an electropore and the possibility of imaging them by any form of microscopy remain unresolved.[8,10,11,13,14]

Electrical exposures typically involve square wave or exponential electric field pulses which generate transmembrane potentials of approximately 1 V and last 10 μs to 10 ms.[8–12] For lipid bilayers on the order of 10 nm thickness, this corresponds to a local field strength within the membrane of 10^6 V/cm. Based largely on electrical measurements, electropores are thought to be created on the submicrosecond time scale.[15–18] They then continue to grow in size for the duration of the electrical exposure. Maximum pore diameters are believed to occur up to 10 nm, although a distribution in sizes is expected.[19] After the pulse, pores are believed to shrink to a metastable state over a characteristic time of milliseconds.[20,21] These long-lived metastable pores are thought to be ~1 nm in radius.[22,23] Having lifetimes from subseconds to hours, these pores eventually disappear completely under reversible conditions. The onset of electroporation has been shown to occur largely independent of exact membrane composition and experimental conditions. However, the time scale of recovery is a strong function of conditions, especially temperature, where low temperature (i.e., 4°C) increases pore lifetimes.[8,10]

Although electroporation has been demonstrated under a variety of conditions, a range of electrical parameters exists for which electroporation is known to occur, and a smaller range exists for which electroporation is reversible. Both the magnitude and duration of the induced transmembrane voltage are important to the occurrence of electroporation. For example, electroporation generally occurs for short pulses (0.1 to 10 μs) which generate a transmembrane voltage slightly greater than 1 V, medium-length pulses (10 to 100 μs) of 0.5 to 1 V, and long pulses (≥1 ms) of 0.2 to 0.5 V.[24,25] Less work has been done on pulses shorter than 0.1 μs.

While electrical characterization of electroporation is important to mechanistic understanding, most applications have emphasized the ability of electroporation to increase molecular transport across lipid bilayers. Many different molecules have been transported across membranes by electroporation, ranging progressively in size from small ions to sugars to oligonucleotides to proteins to DNA to virus particles.[11] Electroporation has found widespread application in molecular biology as a method to introduce DNA into cells in suspension for gene transfection.[8,10] More recently, electroporation of cells in monolayers[26,27] and cells which are part of intact tissues[28–32] also was demonstrated. For example, electroporation of tumors as a method of increasing local cellular uptake of chemotherapeutic agents was applied recently to humans as part of a phase I trial in France.[33] The studies discussed in this chapter also deal with electroporation of tissue, namely skin.[7,34–38] However, in distinct contrast

with other tissues in which cell membranes are electroporated, electroporation of skin appears to involve electroporation of the multilamellar, intercellular lipid bilayers of the SC.

III. SKIN ELECTROPORATION

This chapter discusses whether electroporation of the SC is possible, and whether it can be distinguished from conventional iontophoresis. Although both electroporation and iontophoresis involve electric fields, the two phenomena are fundamentally different. Iontophoresis acts primarily on the drug, moving molecules across skin by electrophoresis and/or electroosmosis. Any skin structural changes are a secondary effect. In contrast, electroporation is expected to cause transient, but large changes in tissue permeability, involving significant structural changes in the skin. The electric field is believed to cause transport by a combination of two mechanisms during electroporation: (1) electropores are created and (2) as pores appear, molecules are moved through the pores by electrophoresis and/or electroosmosis due to the local field.

A. MOLECULAR FLUX

To determine whether electroporation of the SC occurs, we subjected human cadaver epidermis under physiological conditions to electric pulses which cause electroporation in other systems. The experimental methods used were described previously.[7,38] Briefly, heat-stripped cadaver epidermis was loaded into side-by-side permeation chambers, exposed to well-stirred phosphate-buffered saline (PBS, pH 7.4), and allowed to hydrate fully (12 to 18 h, 4°C). The temperature was raised to 37°C and fresh PBS containing 1 mM fluorescent compound (calcein, Lucifer Yellow, or erythrosin derivative) was added to the outer, SC side. After a few hours, electric pulsing was applied with Ag/AgCl electrodes. An exponential pulse (decay time constant, $\tau=1.0$ to 1.3 ms) was applied every 5 s for 1 h, with the negative electrode on the SC side unless otherwise noted. In presenting results we reported voltages across the skin rather than voltages across the electrodes because they are more relevant. The receptor compartment was sampled periodically by emptying its contents and replacing it with fresh PBS. Analysis by calibrated spectrofluorimetry allowed measurement of receptor compartment fluorescent compound concentrations, and thereby, calculation of time-average transdermal fluxes.

Quantitative measurements of transdermal molecular fluxes and electrical measurements are consistent with the three characteristic features of electroporation:[8-12] (1) large increases in molecular flux and ionic conductance, (2) reversibility over a range of voltages, where recovery has two time constants (millisecond and minute), and (3) structural changes in the membrane barrier.

First, transdermal fluxes of calcein (623 Da, − 4 charge), a moderate-sized, highly polar, fluorescent molecule which does not normally cross skin in detectable quantities, were measured during application of low duty cycle electric field pulses. Fluxes before pulsing were below the detection limit (imposed by background fluorescence), while fluxes during pulsing were up to 10,000-fold greater. Figure 1 shows that flux increased nonlinearly with increasing pulse voltage; i.e., the flux increased strongly with increasing voltage below ~100 V and increased weakly with
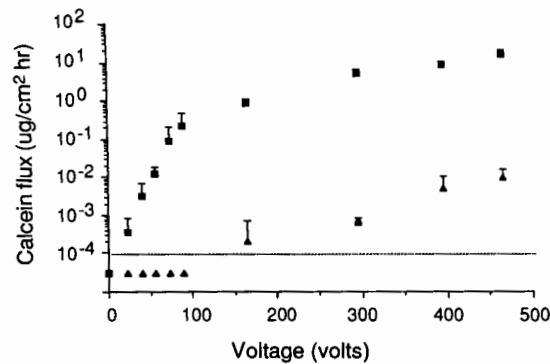
Figure 1 Transdermal fluxes of calcein due to exposure of human skin to different electrical conditions. Calcein flux during application of "forward-polarity" pulses (■) and approximately 1 h after pulsing in the "reverse" direction (see text) (▲). This figure suggests that a transition point may exist at ~100 V, below which flux increases as a strong function of voltage and flux increases are reversible, and above which flux increases only weakly with voltage and effects are only partially reversible. Standard deviation bars are shown. Fluxes below the calcein flux detection limit of 10^{-4} µg/cm²/h are indicated below the dashed line. (From Prausnitz, M. R. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 10504, 1993. With permission.)

increasing voltage at higher voltages. Supporting electrical measurements also showed increases in skin conductance of one to three orders of magnitude (see below).

Second, reversibility was assessed. Following electrical pulsing for 1 h, transdermal fluxes generally decreased by ~90% within 30 min and >99% within 1 or 2 h, consistent with significant reversibility. Electrical conductance measurements also showed recovery (see below). However, elevated postpulsing fluxes could be caused not only by irreversible alterations of skin structure, but also by the efflux of calcein "loaded" into the skin during high fluxes caused by pulsing.

The results of an additional, and possibly better, test of reversibility are also shown in Figure 1. Skin was pulsed with the electrode polarity reversed, leaving the transtissue voltage magnitude during pulsing the same. However, the reversed polarity electrophoretic driving force associated with the pulse should have moved calcein away from the skin, significantly reducing transdermal transport during pulsing. By measuring fluxes ~1 h after such reversed pulsing, long-lived changes in skin permeability can be assessed independently (Figure 1). These data suggest that pulses below ~100 V caused no detectable long-lived changes in skin permeability. However, higher voltage pulses appear to have caused lasting changes. Figure 1 also suggests that a transition region may exist at ~100 V, below which flux increased as a strong function of voltage, and flux increases were reversible; above which flux increased only weakly with voltage and effects were only partially reversible. The exact mechanism underlying this transition is presently unclear. However, for applications it is potentially important that up to 1000-fold flux increases which appear to be fully reversible can be achieved using pulses below ~100 V. The longer-lived changes associated with up to 10,000-fold flux increases may limit application of higher-voltage electroporation.

Third, changes in skin structure cannot be expected to be revealed by microscopy, for reasons discussed below. However, demonstrating that increased fluxes caused
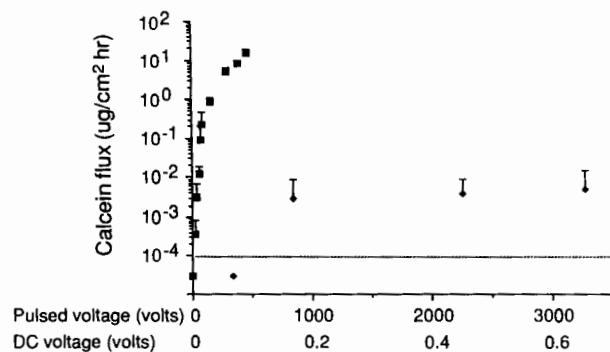
Figure 2 Transdermal fluxes of calcein during pulsing (■) and during application of DC iontophoresis (♦). Upper axis indicates pulsing voltages electrically "equivalent" to continuous DC voltages on lower axis (see text), suggesting that skin structural changes may be needed to explain the high fluxes caused by electroporation. Standard deviation bars are shown. Fluxes below the calcein flux detection limit are indicated below the dashed line. (From Prausnitz, M. R. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 10504, 1993. With permission.)

by pulsing cannot be explained by electrophoresis alone suggests that changes in skin structure are necessary to explain our results. We therefore compared fluxes caused by low duty cycle high-voltage pulsing to fluxes caused by the continuous low-voltage DC current which would provide the same total electrophoretic transport contribution if no changes in skin structure occurred. For example, if the skin were unaltered (i.e., same conductance), then constant application of 0.1 V would transfer the same amount of charge across the skin as the pulsed application of 500 V for 1 ms every 5 s, making these conditions "equivalent" electrophoretically. As seen in Figure 2, application of continuous voltages caused fluxes three orders of magnitude smaller than pulsing under "equivalent" conditions, suggesting that skin structural changes are needed to explain these results.

To appropriately characterize electroporation, we believe that measurement of changes in molecular flux and electrical properties is the best approach, because these measures are widely used in the electroporation literature. Upon initial consideration, electron microscopy might also appear to be an appropriate tool for visualizing the pores created by electroporation. However, currently no satisfactory electron micrographs of electropores in any membrane exist, primarily because electropores are believed to be small (<10 nm), sparse (<0.1% of surface area), and generally short-lived (μs to s). Thus, visualization of electropores by any form of microscopy is not expected.[14] Moreover, although the name "electroporation" suggests the creation of physical pores, all that has been experimentally established is that transiently elevated transport and electrical conductance occur. We therefore did not employ electron microscopy to look for pores in the complex multilaminate structures of the skin, as they have not been imaged in simpler systems.

Enhanced transport of two other polar molecules across the skin was achieved by electroporation: Lucifer Yellow (457 Da, −2 charge) and an erythrosin derivative (1025 Da, −1 charge), a small macromolecule, neither of which normally crosses skin at detectable levels. These molecules were selected because they are fluorescent and have different physical properties than calcein. As seen in Figure 3, pulsing can cause
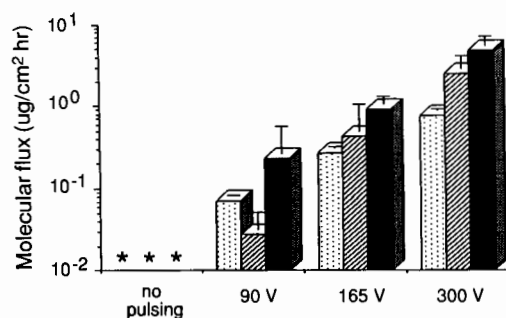
Figure 3 Transdermal fluxes of (▣) an erythrosin derivative (1025 Da, −1 charge), (▨) Lucifer Yellow (457 Da, −2 charge), and (■) calcein across human skin *in vitro*. This figure demonstrates that electroporation increases the flux of a number of polar molecules having different molecular characteristics. Standard deviation bars are shown. The (*) symbol indicates a flux below the detection limit: 10^{-2} µg/cm²/h for the erythrosin derivative and 10^{-3} µg/cm²/h for Lucifer Yellow. (From Prausnitz, M. R. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 10504, 1993. With permission.)

fluxes of both molecules similar to those caused for calcein under the same conditions. This suggests that electroporation-enhanced transport may be broadly applicable to many molecules, possibly including those of larger molecular weights.

Finally, electroporation *in vivo* was performed on anesthetized hairless rats. Using protocols similar to those employed *in vitro,* electroporation at voltages ranging from 30 to 300 V caused transport of 10 to 20 µg/cm²/h.[7,38] No calcein was detected in the serum of unpulsed rats. That the *in vivo* fluxes did not increase with voltage suggests that a rate-limiting step other than transport across the SC existed, perhaps uptake of calcein from a skin depot into the bloodstream. No visible skin damage was observed after pulsing at voltages <150 V; erythema and edema were evident at higher voltages. Long-term biochemical and pathological studies are needed.

Together these results have implications for understanding mechanisms of skin electroporation and for applications to transdermal drug delivery. First, the three characteristic features of electroporation were found in pulsed skin, suggesting that electroporation is the mechanism of flux enhancement. Moreover, for applications, the marked flux increases which are reversible over a range of voltages could make possible the therapeutic delivery of many drugs across skin.

B. ELECTROPORATION AND IONTOPHORESIS

Studies have been performed by Tamada, Bommannan, and co-workers to assess flux increases due to iontophoresis following a single electroporation pulse and to compare them to fluxes by iontophoresis alone.[35,36] The experimental apparatus used was similar to that discussed above, involving heat-stripped human cadaver epidermis in side-by-side permeation chambers with saline buffered at pH 7.4. For electroporated samples, a single exponential-decay electric field pulse was applied (300 to 400 V, $\tau = 5$ to 9 ms), followed by 10 to 60 min constant-current iontophoresis using Ag/AgCl electrodes. Passive flux was measured before and after electrical exposures. For iontophoresis-only control samples, the identical protocol was followed, except the high-voltage pulse was omitted.

The results of a study with luteinizing hormone-releasing hormone (LHRH, 1182 Da, +1 net charge) are shown in Figure 4. For iontophoresis-only samples, the flux
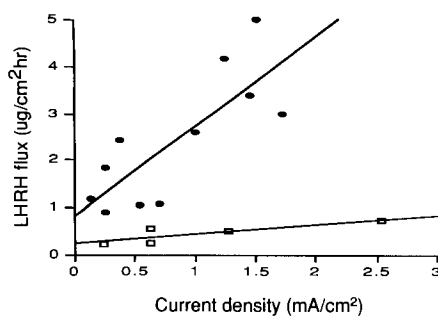
Figure 4 Transdermal transport of LHRH due to iontophoresis following a single electroporation pulse (•) and iontophoresis alone (□). See text for experimental protocols. Linear regressions are shown. This figure shows that application of a single electroporation pulse before iontophoresis can significantly increase the flux of LHRH relative to iontophoresis alone. (Data from Bommannan, D. et al., *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, 20, 97, 1993.)

increased with increasing current density. However, for the samples exposed to iontophoresis at the same current density, following a single electroporation pulse, LHRH was transported to a significantly greater extent. Moreover, within several hours after the electrical exposure, fluxes decreased substantially and were close to pretreatment levels. Similar results have been shown for [arg[8]]-vasopressin (1084 Da, +2 net charge) and neurotensin (1693 Da, +1 net charge), in which flux increases due to electroporation followed by iontophoresis were three to eight fold greater than iontophoresis alone.

These results demonstrate that the enhancement due to electroporation and iontophoresis can be combined, making an even more powerful approach to transdermal drug delivery than either one alone. Moreover, the additional enhancement of peptide transport across the skin due to electroporation may lead to noninvasive, controlled delivery of peptides at therapeutic levels. These results show that a single electroporation pulse increased the effects of iontophoresis for many minutes after the pulse, but appeared to reverse after hours. This indicates that a lasting change in skin properties occurred and is consistent with transient structural changes.

C. ELECTRICAL ANALYSIS

Skin electrical properties have been extensively characterized.[39–42] Changes in skin impedance have been reported in response to a wide range of physical and emotional stimuli, exhibiting significant intersubject variability. The most commonly used electrical model for skin is the parallel combination of a resistor (~100 kΩ cm^2) and a capacitor (~10 nF/cm^2). More complicated models were proposed in which the choice of model is determined by the skin substructures of interest and the relevant frequency range. However, these linear models are valid only over a small voltage range (<1 V), above which the electrical properties of skin are nonlinear.[43–45]

To study changes in skin impedance due to electroporation, skin was prepared and loaded into permeation chambers as in the molecular flux experiments. Measurement and analysis of skin electrical properties were done as described previously.[37] Briefly, an exponential voltage pulse was applied across the chamber. Current during the pulse was obtained by measuring the voltage across a 5-Ω sampling resistor placed in series with the chamber, while the voltage across the skin was measured using two

Ag/AgCl electrodes placed near the skin. Impedance spectra were measured before and after pulsing by applying current steps ranging from 0.1 to 2 µA to the outer electrodes of a four-electrode measurement system. The voltage developed at the inner electrodes was measured by a high impedance differential amplifier and digitized by a PC-based data acquisition system. The data were later analyzed to determine the skin impedance as a function of frequency. The impedance spectra were then fit to the most statistically significant electric circuit model, which consisted of the typical parallel R-C circuit, modified by placing a series R-C circuit in parallel with it. The series R-C is a lumped approximation of the high-frequency properties of skin. This approximation provides a more accurate representation of skin electrical properties over the relevant frequency range (less than a few kilohertz).[37] Impedance measurements were made before the pulse and at several times >20 ms after the pulse.

The saline on either side of the chamber had a resistance on the order of 100 Ω. Thus, the nominal charging time of the system (in the absence of nonlinear effects) would be on the order of 1 µs (i.e., charging time, $\tau = RC = 100\ \Omega \times 10\ \text{nF} = 1$ µs). If skin electrical properties remained unchanged during a pulse (i.e., $R = 10^5\ \Omega$ and $C = 10$ nF), current after the microsecond charging time would be 1 to 5 mA for typical pulses of 100 to 500 V across the skin. However, currents of 500 to 4000 mA were measured under these conditions (data not shown). The three order of magnitude difference between predicted and measured currents suggests that skin electrical properties underwent dramatic changes on the time scale of a microsecond. This result is supported by measurements made near the end of the pulse, where effective skin resistances on the order of 100 Ω were determined. This was done by assuming a linear circuit model is valid because the voltage is nearly constant at the end of the pulse.

For transdermal voltages <100 V, the impedance spectrum measured 20 ms after the pulse was identical to the prepulsing impedance. This suggests that within milliseconds the skin had recovered fully from the changes induced during pulsing. At higher voltages a threshold appeared, above which lasting changes in impedance were evident. This threshold was between 200 and 300 V, depending on the particular sample. Figure 5A shows the impedance of a representative skin sample before pulsing, and at three different times after pulsing. The most significant change in the impedance was the resistance (impedance at 0 Hz), which was determined by evaluating the DC impedance of the fitted model. Resistance after a pulse is plotted as a function of time in Figure 5B. This curve has three characteristic time constants: one <20 ms (between the pulse and the first impedance measurement), one between 5 and 20 s, and the third between 50 and 200 s. Resistance returned to 5 to 100% of its prepulse value, depending on pulsing voltage and the sample used. Incomplete recovery indicates that some permanent changes in skin structure occurred.

These results are consistent with three important features of electroporation:

1. Changes in skin electrical properties of several orders of magnitude occurred on a time scale of microseconds. Although changes in skin electrical properties also occur in iontophoresis (i.e., exposure at a few volts over longer times), skin resistance decreases by only up to one order of magnitude on the time scale of minutes. This is a seven order of magnitude difference in time scale.
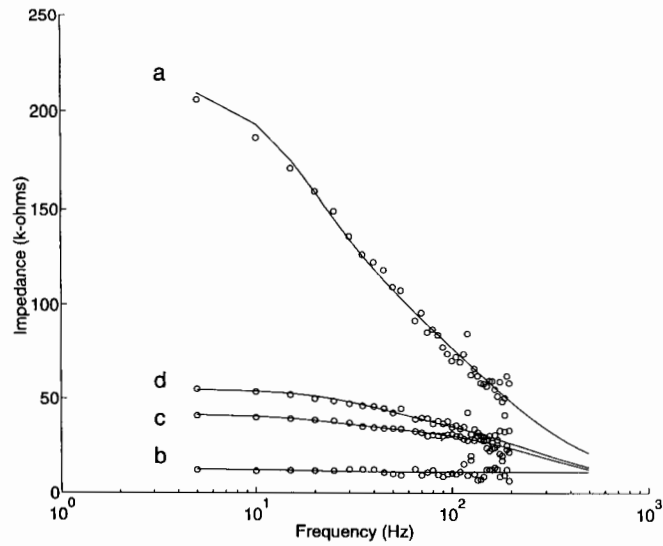
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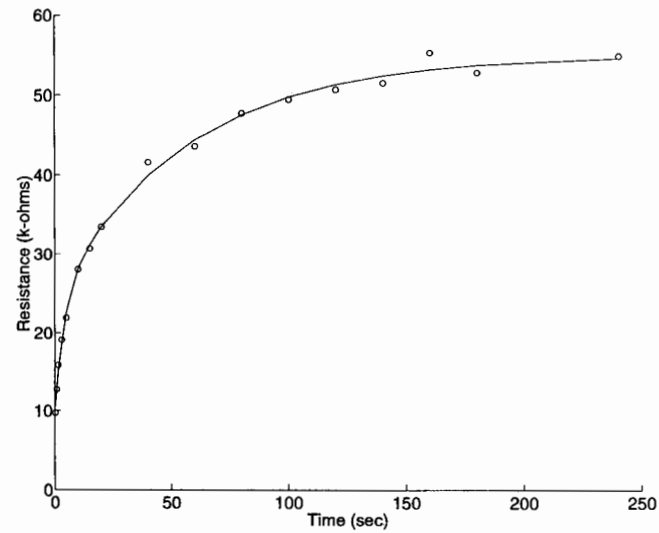
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Figure 5 (A) Impedance spectra of a representative skin sample before pulsing at 210 V (a), and at 800 ms (b), 40 s, (c) and 4 min (d) after pulsing. The impedance "recovered" toward its prepulse value over time. (B) Resistance of the skin after pulsing, calculated from impedance spectra. Prepulse resistance was 220 kΩ; 20 ms postpulse the resistance was 20-fold lower. The resistance recovery was characterized by three time constants: <20 ms (between the pulse and the first impedance measurement), 5 s, and 57 s for the data shown.

2. Apparently, a threshold of a few hundred volts exists, below which long-lasting electrical changes in skin are not seen. Assuming a few hundred bilayers lie in a cross-section of SC, the observed electroporation threshold of a few hundred volts would be expected because the literature shows that the electroporation threshold for a single lipid bilayer is on the order of 1 V.[8-12]

3. A range of voltages exists over which changes in electrical properties are fully reversible and above which the changes are only partially reversible. The existence of these two ranges is consistent with electroporation in other systems.[8-12]

IV. DISCUSSION

A. ELECTROPORATION MECHANISM

It is well established that the SC is the primary barrier to transdermal transport;[1-4] thus, our interpretation is that changes in the SC account for the observed increases in flux due to electroporation. Although studied mainly in the context of living cells, electroporation also has been investigated widely in artificial planar bilayer membranes and liposomes.[8-12,23] Because electroporation is a physical process based on electrostatic interactions and thermal fluctuations within fluid membranes, no active transport processes are involved.[8-12] Thus, electroporation could occur in the SC, even though it does not contain living cells.

"Proving" that electroporation occurs in skin cannot be done. In the literature electroporation is described experimentally as characteristic behavior (e.g., very large increases in molecular transport and conductance in lipid bilayers), occurring at characteristic voltages (e.g., approximately 1 V across a bilayer), and over characteristic times (e.g., submicrosecond onset and biphasic recovery over milliseconds and minutes). Although the mechanisms by which these events occur (e.g., creation of aqueous pores, called electropores) are plausible, these mechanisms are hypotheses; electropores have not been experimentally observed by imaging. Therefore, an experimental investigation of skin electroporation should establish whether phenomena similar to those observed in cell electroporation occur in skin as well.

The above experiments demonstrate very large increases in transdermal flux, which are reversible over a range of conditions and appear to be associated with structural changes. These results were seen with six different molecules having molecular weights up to approximately 1700 Da. Similar results were observed *in vivo* with animal skin. Electrical analysis showed dramatic electrical changes occurring within 1 μs and recovery with millisecond and second time constants. Our interpretation is that these experimental results exhibit the characteristic behavior of electroporation. We therefore conclude that electroporation of skin has occurred.

B. DRUG DELIVERY APPLICATIONS

Although electroporation causes large flux increases across the SC, deeper viable tissue may be essentially unaffected. This localization is expected because the SC has a much higher electrical resistance than other regions of the skin. As a result, an electric field applied to the skin will concentrate in the SC, resulting in other, viable tissues being exposed to much lower fields. Therefore, an electric field sufficient to cause electroporation could exist in the SC, while a significantly lower field exists in viable tissues, insufficient to cause electroporation. An implicit targeting mechanism results in which the greatest electric fields are generated where the largest

resistivities exist, thereby protecting the already-permeable viable parts of the skin and deeper tissues.

It is presently difficult to state with certainty which electrical conditions will be acceptable for clinical use. Many features, including pulse voltage/current/energy, pulse length, pulse frequency, duration of total exposure, and electrode size, site, and design, will be important. A complete consideration of the safety of electroporation of skin is beyond the scope of this study. However, that the electrical exposures used were fully reversible over a range of voltages is a strong indication that the procedure is not damaging and may be safe. Moreover, a clinical precedent has been set for safely applying electric pulses to skin with voltages up to hundreds of volts and durations up to milliseconds. Such diagnostic and therapeutic applications include transcutaneous electrical nerve stimulation, functional electrical stimulation, electromyography, and somatosensory evoked potential testing.[46,47]

Because of the overall hydrophobic character and net negative charge of the SC, transdermal transport of negatively charged hydrophilic molecules is especially challenging.[1-4] Calcein, with eight charge sites and a net charge of -4,[48] is therefore considerably more difficult to transport across the skin than many other molecules. Approaches to transdermal flux enhancement involving chemical enhancers have been successful with some lipophilic and moderately polar molecules, but limited in applicability to highly polar and charged molecules. Iontophoresis was employed successfully with some polar and charged molecules. For many drugs, delivery rates in the microgram per square centimeter per hour range could be therapeutic, while significantly higher rates of delivery may be required for other drugs. In general, a 10-fold increase in flux caused by an enhancement method is impressive, while a 100-fold increase is of great interest. Thousand-fold increases are rarely found. The up to 10,000-fold increases in flux caused by electroporation are therefore potentially very significant and could make possible transdermal delivery of many drugs at therapeutic levels.

Finally, transdermal flux enhancement has been demonstrated with other techniques, including chemical, iontophoretic, and ultrasonic methods. Because electroporation is mechanistically different, involving temporary alterations of skin structure, it could be used in combination with these or other enhancers. Together, these results suggest that electroporation of skin occurs and may be useful to enhance transdermal drug delivery.

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