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Electroporation of mammalian skin: A mechanism to enhance transdermal drug delivery

(stratum corneum/lipid bilayer/electropermeabilization/tissue/iontophoresis)

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ABSTRACT **Mammalian skin owes its remarkable barrier function to its outermost and dead layer, the stratum corneum. Transdermal transport through this region occurs predominantly through intercellular lipids, organized largely in bilayers. Electroporation is the creation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) electric pulse. Our measurements suggest that electroporation occurs in the intercellular lipid bilayers of the stratum corneum by a mechanism involving transient structural changes. Flux increases up to 4 orders of magnitude were observed with human skin *in vitro* for three polar molecules having charges between −1 and −4 and molecular weights up to slightly more than 1000. Similar flux increases were observed *in vivo* with animal skin. These results may have significance for drug delivery and other medical applications.**

Transdermal drug delivery offers a number of potential advantages over conventional methods, such as pills and injections: (*i*) no degradation due to stomach, intestine, or first pass of the liver, (*ii*) probable improved patient compliance because of a user-friendly method, and (*iii*) potential for steady or time-varying controlled delivery (1–4). Nevertheless, very few drugs can be administered transdermally at therapeutic levels, due to the low permeability and lipophilic nature of human skin. As a result, fewer than 10 drugs are now clinically administered transdermally. However, the market for these drugs exceeds one billion dollars in the United States alone, indicating the importance of this delivery method. Therefore, significant enhancement of transdermal drug delivery has the potential for major impact on medicine.

A number of approaches have been taken to increase transdermal transport (2–4). Most common is the addition of chemical enhancers, compounds which are believed to increase the partitioning of drugs into the skin. Another approach is chemical modification of a drug into a "prodrug," which penetrates the skin well but is subsequently converted by epidermal enzymes into the original pharmacologically active drug. Application of ultrasound has been used as well to increase transdermal flux and to reduce transport lag times. Yet another approach is iontophoresis, the movement of drugs across the skin by an electric field. Mechanistically similar to electrophoresis, iontophoresis is believed to act primarily by moving charged species across the skin by an electrical force.

The barrier properties of skin are attributed primarily to the stratum corneum, the skin's outer layer. The stratum corneum is a dead tissue composed of flattened cells filled with crosslinked keratin and an extracellular matrix made up of lipids arranged largely in bilayers (5, 6). Unlike the unilamellar phospholipid bilayers of cell membranes, these multilamellar, extracellular bilayers contain no phospholipids, being composed primarily of ceramides, cholesterol, and fatty acids (1–3). Intercellular pathways are generally the most important routes for transdermal transport (1–3). Therefore, permeabilization of the lipid bilayers filling these intercellular pathways would be expected to increase transdermal transport.

Electroporation is a method of reversibly permeabilizing lipid bilayers, involving the creation of transient aqueous pores by the application of an electric pulse (7, 8). Dramatically reduced electrical resistance and extensive transport of molecules, including macromolecules, are generally associated with electroporation of lipid bilayers, including membranes of artificial planar and spherical systems, as well as those of living cells. Electric field exposures causing electroporation typically generate transmembrane potentials of $\approx$1 V and last 10 μsec to 10 msec. Electroporation of isolated single cells is well established, but electroporation of cells that are part of an intact tissue has received little attention (9–12). To our knowledge, electroporation of multilamellar or non-phospholipid systems has not been previously demonstrated.

In this study, we examine the possibility of electroporating the multilamellar, non-phospholipid, intercellular lipid bilayers of the stratum corneum as a mechanism to enhance transdermal drug delivery. Although both electroporation and iontophoresis involve electric fields, the two approaches are fundamentally different. While iontophoresis acts primarily on the drug, involving skin structural changes as a secondary effect (2, 3), electroporation is expected to act directly on the skin, making transient changes in tissue permeability. Because electroporation of cells has been shown to increase transmembrane fluxes dramatically and reversibly, electroporation of skin could make possible the transdermal delivery of many more drugs at therapeutic levels.

MATERIALS AND METHODS

Materials. Phosphate-buffered saline (PBS) was prepared containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.1 mM KH_2PO_4 (Mallinckrodt) and was adjusted to pH 7.4 by adding NaOH or HCl (Mallinckrodt). Calcein was obtained from Sigma and Molecular Probes. Lucifer yellow and erythrosin-5-iodoacetamide were obtained from Molecular Probes. To make the sulfur-alkylated erythrosin derivative, erythrosin-5-iodoacetamide was incubated with excess 6-mercapto-1-hexanol in PBS at 25°C for >12 hr.

Skin Preparation. By use of established methods for skin sample preparation, full-thickness excised cadaver skin was obtained within 48 hr after death and stored at 4°C and 95% humidity for up to 1 week (2, 3). Full-thickness samples were prepared by gently scraping off subcutaneous fat. Epidermis samples were heat separated by submerging full-thickness

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skin in 60°C water for 2 min and gently removing the epidermis (13). All samples were stored at 4°C and 95% humidity for <3 weeks. Tissue was obtained from four sources (three local hospitals and the National Disease Research Interchange) to minimize any artifacts of tissue acquisition. Tissue was generally from the abdomen, removed just lateral to the midline, although tissue from the breast, back, and thigh have been used as well.

Because the primary barrier to transport is the stratum corneum (the upper 10–20 μm of the epidermis), the use of epidermis rather than full-thickness skin is a well-established model for transdermal drug delivery (2, 3). In the literature, transdermal drug delivery is commonly understood to mean transport of drugs across the skin (not just the dermis) (1–4). When systemic delivery is desired, a drug must traverse the stratum corneum, the viable epidermis, and some fraction of the dermis before entering blood vessels of the systemic circulation. Since capillaries exist near the dermal/epidermal junction, drugs can enter the systemic circulation without crossing the whole dermis (1–3). Thus, transport across full-thickness skin misrepresents the actual transport pathway. For these reasons, following established practice (2, 3), we have performed the majority of our studies with human epidermis and have established agreement with select results from full-thickness human skin.

***In Vitro* Methods.** Prepared skin samples were loaded into side-by-side permeation chambers (14), exposed to well-stirred PBS, and allowed to hydrate fully (12–18 hr, 4°C). The temperature was raised to 37°C and fresh PBS was added, with 1 mM fluorescent compound (calcein, Lucifer yellow, or erythrosin derivative) on the outer, stratum corneum side. After a steady-state flux was established (within a few hours), electric pulsing was applied with Ag/AgCl electrodes (≈2 cm from skin) (In Vivo Metrics, Healdsburg, CA). An exponential-decay pulse (τ = 1.0–1.3 msec; Gene-Pulser; Bio-Rad) was applied every 5 sec for 1 hr, with the negative electrode on the stratum corneum side ("forward" pulsing), except for "reverse" pulsing, where the positive electrode was on the stratum corneum side. Iontophoresis (continuous dc voltage) was also used for 1 hr, with the negative electrode on the stratum corneum side. The receptor compartment was sampled periodically by emptying its contents and replacing it with fresh PBS. Analysis by calibrated spectrofluorimetry (Fluorolog-2, model F112AI; Spex Industries, Metuchen, NJ) allowed measurement of fluorescent compound concentrations in the receptor compartment and, thereby, calculation of transdermal fluxes.

Reported voltages are transdermal values, determined at >1 μsec after the onset of the pulse. During a pulse, the apparent resistance of the chamber, without skin (but including electrodes, saline, and interfacial resistances), was 480 Ω, independent of the pulse voltage. The apparent resistance of the chamber with skin varied from 900 Ω during lower-voltage pulses (≈50 V across skin) to 600 Ω during higher-voltage pulses (≈500 V across skin), meaning that only 20–50% of the applied voltage appeared across the skin. Transdermal voltages were determined by calculating the ratio of the apparent skin resistance to the apparent total chamber (with skin) resistance. This ratio is equal to the ratio of the transdermal voltage to the voltage across the whole chamber (with skin). By application of a voltage pulse and measurement of the resulting current, apparent resistances were calculated by dividing the applied voltage by the measured current.

Post-pulse skin electrical characterization was performed with a four-electrode impedance-measurement system. A current step (2 μA/cm^2) was applied and the resulting transdermal voltage was measured. By using a Fourier transform, skin impedance was calculated over a range of frequencies (1–1000 Hz) by dividing the measured transdermal voltage by the applied current.

***In Vivo* Methods.** For *in vivo* studies, reservoirs (≈4 ml, 2.8 cm^2) with Ag/AgCl electrodes (≈1 cm from skin) were attached to gently pinched skin from the caudal dorsal region of anesthetized (ketamine, 75 mg/kg, and xylazine, 10 mg/kg, with additional one-third doses given every 30–45 min) CD hairless rats (Charles River Breeding Laboratories) (12); animal care was in accordance with institutional guidelines. Hairless rodents are commonly used as *in vivo* models for transdermal studies (2, 3). Both reservoirs were filled with PBS; the negative electrode side contained 10 mM calcein. Pulses were applied as described above for 1 hr.

Plasma calcein concentration measurements were made 30–60 min after pulsing. Blood samples were taken from the lateral tail vein, transferred into a serum separator tube (Microtainer, Becton Dickinson), and spun at 1000 × *g* for 5 min to isolate the plasma for analysis by calibrated spectrofluorimetry. 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 did not occur over that period (15, 16). The volume of distribution determined from these measurements was 20% of total rat volume (17), which is equal to the volume of the extracellular aqueous compartment (18). Given the very hydrophilic nature of calcein (19), distribution throughout all extracellular aqueous regions is a reasonable assumption.

RESULTS AND DISCUSSION

To determine whether electroporation of the stratum corneum was possible, we subjected human cadaver epidermis under physiological conditions to electric pulses which cause electroporation in other systems. Quantitative measurements of transdermal molecular flux, supported by electrical measurements, are consistent with three unique characteristics of electroporation (7, 8): (*i*) large increases in molecular flux and ionic conductance, (*ii*) reversibility over a range of voltages, and (*iii*) changes in barrier membrane structure.

First, transdermal fluxes of calcein (623 Da, −4 charge), a moderate-sized, highly polar molecule which does not normally cross skin in detectable quantities, were measured during application of low-duty-cycle electric-field pulses. Fig. 1 shows average transdermal fluxes of calcein before, during, and after pulsing at representative voltages. Fluxes before pulsing were below the detection limit (imposed by background fluorescence), whereas fluxes during pulsing were up to 4 orders of magnitude above this limit. Fig. 2 shows that flux increased nonlinearly with increasing pulse voltage, where flux increased strongly with increasing voltage below ≈100 V and increased weakly with increasing voltage at higher voltages. Supporting electrical measurements also showed increases in skin conductance of 1–3 orders of magnitude. Electrical changes were evident during the pulse and immediately (≥10 msec) after. This is consistent with changes caused by electroporation, the onset of which is believed to occur on the microsecond scale (20–22).

Second, reversibility was assessed. Following electrical pulsing for 1 hr, transdermal fluxes generally decreased by about 90% within 30 min and by >99% within 1 or 2 hr (Fig. 1), indicating significant reversibility. Electrical conductance measurements also showed recovery, either complete or to within a factor of 3 of pre-pulse values. However, elevated post-pulsing 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 during pulsing (23, 24).

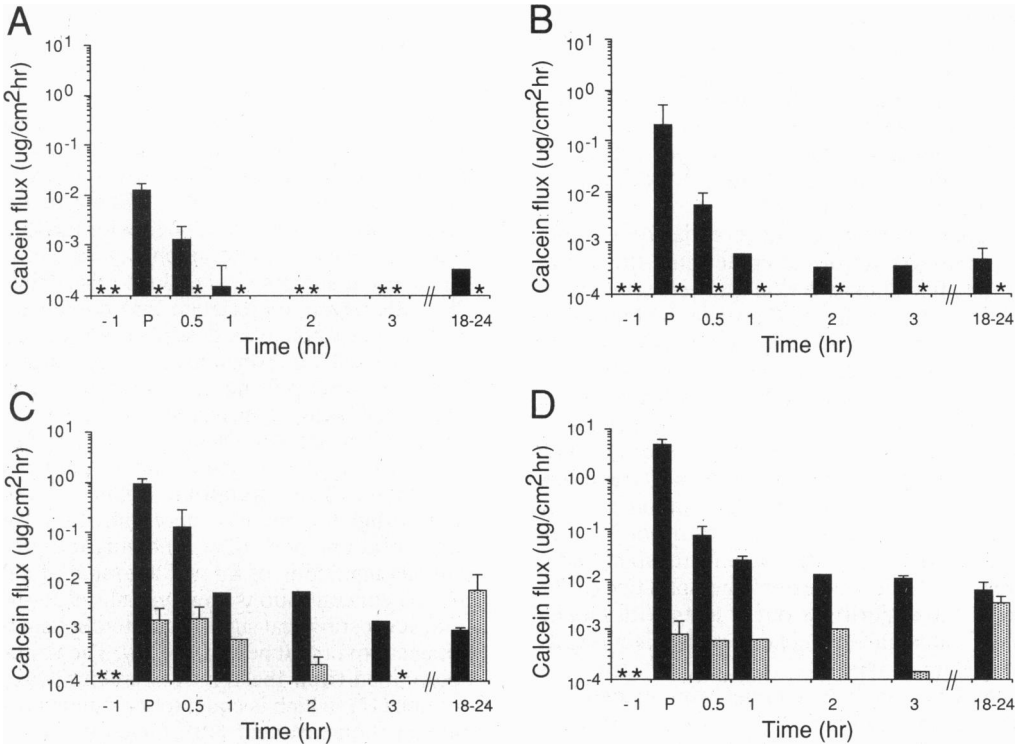

FIG. 1. Transdermal fluxes of calcein (623 Da, −4 charge) before, during, and after "forward" pulsing (solid bars) or "reverse" pulsing (stippled bars) at 55 V (*A*), 90 V (*B*), 165 V (*C*), and 300 V (*D*). Flux increases up to 4 orders of magnitude are observed under "forward" pulsing conditions (see text). These increases are at least partially reversible. "Reverse" pulsing facilitates independent assessment of changes in skin permeability due to electroporation (see text), suggesting that skin electroporation may be fully reversible below ≈100 V, under the conditions used. Fluxes are shown 1 hr before pulsing, during pulsing (P), and at times after pulsing. Pulsing was performed for 1 hr (see text). Elevated fluxes at 18–24 hr could be caused by skin deterioration. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit, of order 10^{-4} μg/(cm^2·hr).

The results of an additional, and possibly better, test of reversibility are also shown in Fig. 1: skin was pulsed with the electrode polarity reversed, leaving the transmembrane voltage magnitude during pulsing the same. However, the electrophoresis associated with the pulse should move calcein away from the skin under these conditions, markedly reducing transdermal transport during pulsing. By measuring fluxes ≈1 hr after reverse-pulsing, long-lived changes in skin permeability can be assessed independently, as summarized in Fig. 2. These data suggest that pulses at or below ≈100 V cause no detectable long-lived changes in skin permeability. However, higher voltage pulses appear to cause lasting changes; these changes do not go away, even after 18–24 hr. Fig. 2 also suggests that a transition region 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. The exact mechanism underlying this transition is unclear.

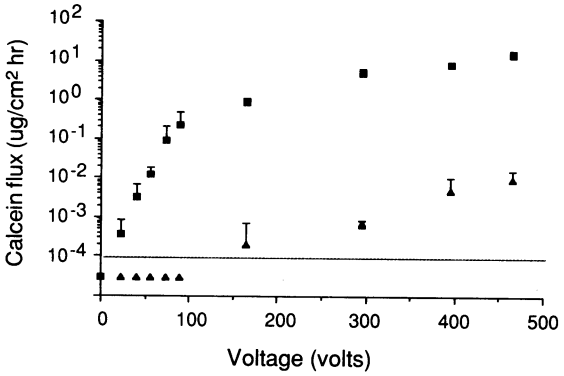

FIG. 2. Transdermal fluxes of calcein due to exposure of human skin to different electrical conditions. Calcein flux during application of forward-polarity pulses (■) and ≈1 hr after pulsing in the reverse direction (see text) (▲). This figure suggests that a transition point may exist at ≈100 V, below 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. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Fluxes below the calcein flux detection limit are indicated below the dashed line.

Third, changes in skin structure cannot be expected to be revealed by microscopy, for reasons discussed below. However, demonstrating that increased fluxes caused 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 component if no changes in skin structure occurred. For example, if the skin were unaltered (i.e., same conductance), then continuous application of 0.1 V would transfer the same amount of charge across the skin as the pulsed application of 500 V for 1 msec every 5 sec, making these conditions electrophoretically "equivalent." As seen in Fig. 3, application of continuous voltages caused fluxes 3 orders of magnitude smaller than pulsing under "equivalent" conditions, suggesting that skin structural changes are needed to explain these results.

To assess the occurrence and reversibility of electroporation, we believe that characterization of flux changes, along with companion electrical measurements, is the best approach, since these measures are universally accepted in the

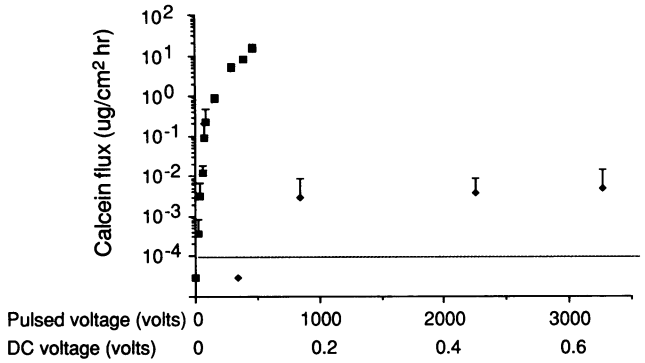

FIG. 3. Transdermal fluxes of calcein during pulsing (■) and during application of dc iontophoresis (◆). Upper axis indicates pulsing voltages electrically "equivalent" to continuous dc voltages on lower axis (see text), suggesting that skin structural changes may be needed to explain the high fluxes caused by electroporation. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Fluxes below the calcein flux detection limit are indicated below the dashed line.

electroporation literature (7, 8). Upon initial consideration, electron microscopy might also appear to be an appropriate tool for visualizing the pores created by electroporation. However, there currently exist no satisfactory electron micrographs of electropores in any membrane, primarily because electropores are believed to be small (<10 nm), sparse (<0.1% of surface area), and generally short-lived (microseconds to seconds) (7, 8). Thus, it is extremely difficult to visualize electropores by electron microscopy. Moreover, although the name electroporation suggests the creation of physical pores, all that has been concretely established is that a transient high-permeability state is created, characterized by greatly increased permeability and electrical conductivity (7, 8). We therefore did not employ electron microscopy to look for pores in the complex multilaminate structures of the skin, since their existence had not been established 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 Fig. 4*A*, pulsing can cause 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 higher molecular weights.

We have also observed flux increases due to pulsing of full-thickness human skin, suggesting that artifacts due to epidermis preparation are not significant. However, full-thickness fluxes were delayed (about 1 hr) and were about an order of magnitude lower, probably due to binding or diffusional limitations in the dermis (2–3 mm thick). Moreover, we have observed up to 1000-fold flux increases due to pulsing in frog and hairless rat skin *in vitro* (data not shown).

Finally, electroporation *in vivo* was performed on hairless rats, assessed by measuring serum concentrations of calcein delivered transdermally (Fig. 4*B*). Fluxes in excess of 10 $\mu g/(cm^2 hr)$ were observed at voltages as low as 30 V; these fluxes are at least 2 orders of magnitude greater than controls (Fig. 4*B*). That the *in vivo* fluxes do not increase with voltage suggests that a rate-limiting step other than transport across the stratum corneum exists, perhaps uptake of calcein from a skin depot into the bloodstream. No visible skin damage

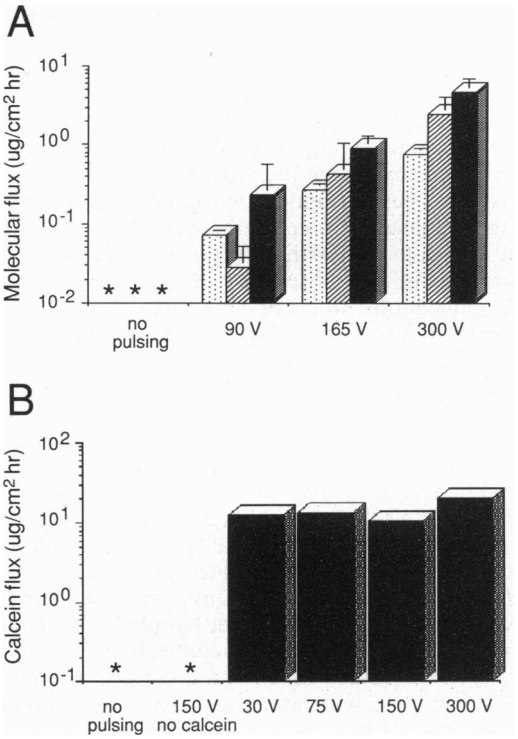

FIG. 4. Transdermal fluxes *in vitro* and *in vivo*. (*A*) Transdermal fluxes of an erythrosin derivative (1025 Da, −1 charge) (stippled bars), Lucifer yellow (457 Da, −2 charge) (hatched bars), and calcein (solid bars) across human skin *in vitro*. This figure demonstrates that electroporation increases the flux of a number of polar molecules having different molecular characteristics. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit, of order 10^{-2} $\mu g/(cm^2 hr)$ for the erythrosin derivative and 10^{-3} $\mu g/(cm^2 hr)$ for Lucifer yellow. (*B*) Flux of calcein across hairless rat skin *in vivo*, which suggests that electroporation can increase transdermal flux in a living animal. Each point represents the average result from one or two rats. Asterisk indicates a flux below the detection limit, of order 10^{-1} $\mu g/(cm^2 hr)$.

was observed after pulsing at voltages below 150 V; erythema and edema were evident at higher voltages. Long-term biochemical and pathological studies are needed.

It is well established that the stratum corneum is the primary barrier to transdermal transport (1–4); thus, changes in the stratum corneum probably account for the observed increases in flux due to electroporation. Although it has been applied primarily to living cells, electroporation has also been widely studied in artificial planar bilayer membranes and liposomes (7, 8). Electroporation is a physical process based on electrostatic interactions and thermal fluctuations within fluid membranes; no active transport processes are involved (7, 8). Thus, electroporation could occur in the stratum corneum even though it does not contain living cells.

The stratum corneum has a much higher electrical resistance than other parts of the skin. As a result, an electric field applied to the skin will concentrate in the stratum corneum, resulting in other, viable tissues being exposed to much lower fields. Therefore, under appropriate conditions, an electric field sufficient to cause electroporation could exist in the stratum corneum, while a significantly lower field existed in viable tissues, insufficient to cause electroporation. An implicit targeting mechanism results, where 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 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 (25). A complete histological examination 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 prove to be safe under appropriate conditions. Moreover, there exists a clinical precedent for safely applying electric pulses to skin with voltages up to hundreds of volts and durations up to milliseconds. Such diagnostic and therapeutic applications, which involve stimulation of nerves and may inadvertently cause electroporation of skin, include transcutaneous electrical nerve stimulation, functional electrical stimulation, electromyography, and somatosensory-evoked-potential testing (25, 26).

Because of the stratum corneum's overall hydrophobic character and net negative charge, transdermal transport of negatively charged hydrophilic molecules is especially challenging (2, 3). Calcein, with eight charge sites and a net charge of −4 (19), 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 are limited in applicability to highly polar and charged molecules (2–4). Iontophoresis has been successfully employed with some polar and charged molecules (2–4). For many drugs, delivery rates in the range of micrograms per square centimeter per hour could be therapeutic, whereas significantly higher rates of delivery may be required for other drugs (2, 3). In general, a 10-fold increase in flux caused by an enhancement method is impressive, and a 100-fold increase is of great interest. Thousandfold increases are rarely found (2, 3). The increases of up to 10,000-fold in flux that are 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 methods, including chemical, iontophoretic, and ultrasonic (2–4). Because electroporation is mechanistically different, involving temporary alterations of skin structure, it could be used in combination with these or other enhancers. Electroporation may also be useful in other applications involving transport across skin, such as noninvasive sensing for biochemical measurement, gene therapy, and cancer chemotherapy. Together, these results suggest that electroporation of mammalian skin occurs and may be useful as a mechanism to enhance transdermal drug delivery.

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