Saturated Anionic Phospholipids Enhance Transdermal Transport by Electroporation

Arindam Sen, Ya-Li Zhao, and Sek Wen Hui
Membrane Biophysics Laboratory, Molecular and Cellular Biophysics Department, Roswell Park Cancer Institute, Buffalo, New York 14263 USA

ABSTRACT Anionic phospholipids, but not cationic or neutral phospholipids, were found to enhance the transdermal transport of molecules by electroporation. When added as liposomes to the milieus of water-soluble molecules to be delivered through the epidermis of porcine skin by electroporation, these phospholipids enhance, by one to two orders of magnitude, the transdermal flux. Encapsulation of molecules in liposomes is not necessary. Dimyristoylphosphatidylserine (DMPS), phosphatidylserine from bovine brain (brain-PS), dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidylglycerol (DOPG) were used to test factors affecting the potency of anionic lipid transport enhancers. DMPS with saturated acyl chains was found to be a much more potent transport enhancer than those with unsaturated acyl chains (DOPS and DOPG). There was no headgroup preference. Saturated DMPS was also more effective in delaying resistance recovery after pulsing, and with a greater affinity in the epidermis after pulsing. Using fluorescent carboxyl fluorescein and fluorescein isothiocyanate (FITC)-labeled Dextrans as test water-soluble molecules for transport, and rhodamine-labeled phospholipids to track anionic phospholipids, we found, by conventional and confocal fluorescence microscopy, that transport of water-soluble molecules was localized in local transport spots or regions (LTRs) created by the electroporation pulses. Anionic phospholipids, especially DMPS, were located at the center of the LTRs and spanned the entire thickness of the stratum corneum (SC). The degree of saturation of anionic phospholipids made no difference in the densities of LTRs created. We deduce that, after being driven into the epidermis by negative electric pulses, saturated anionic phospholipids mix and are retained better by the SC lipids. Anionic lipids prefer loose layers or vesicular rather than multilamellar forms, thereby prolonging the structural recovery of SC lipids to the native multilamellar form. In the presence of 1 mg/ml DMPS in the transport milieu, the flux of FITC-Dextran-4k was enhanced by 80-fold and reached 175 $\mu g/cm^2$/min. Thus, the use of proper lipid enhancers greatly extends the upper size limit of transportable chemicals. Understanding the mechanism of lipid enhancers enables one to rationally design better enhancers for transdermal drug and vaccine delivery by electroporation.

INTRODUCTION

Transdermal drug delivery has many potential advantages over other delivery methods. However, at present, the clinical use of transdermal delivery is limited by the fact that very few drugs can be delivered transdermally at a viable rate. This is because the skin forms an efficient barrier for most molecules, and very few noninvasive methods are known to significantly facilitate the penetration of this barrier. The application of electroporation to transdermal delivery is a relatively recent development (Prausnitz et al., 1993). Electroporation is a method to transiently permeabilize a membrane or layers of membranes by the application of a single or multiple short-duration pulses. If the potential drop exceeds the membrane breakdown potential, pores may form and reseal, depending on the applied pulse field and duration. During the lifetime of the pores, materials may be transported across the membrane.

Currently, only small drug molecules can be transported through the epidermis by electroporation. These include negatively charged (Chen at al., 1998) as well as positively charged fluorescent dyes (Johnson et al., 1998), flurbiprofen (Vanbever and Preat, 1999), fentanyl (Vanbever et al., 1998a), dihydrotestosterone (Clarys et al., 1998), alniditan (Jadoul et al., 1998a), domperidone (Jadoul and Preat, 1997), and modified oligonucleotides (Regnier et al., 2000, Regnier and Preat, 1998). Transport of small neutral molecules such as mannitol (Vanbever et al., 1998b) and glucose (unpublished) was also possible. The molecular mass cutoff was determined, using fluorescein isothiocyanate (FITC)-Dextrans, to be ~10 kDa (Lombry et al., 2000; Sen et al., 2002). Larger molecules, including heparin, polylysine (Vanbever et al., 1997; Weaver et al., 1997), lactalbumin and IgG (Zewert et al., 1999), as well as antigenic peptides (Misra et al., 2000) were delivered by transdermal electroporation mostly with molecular enhancers such as keratolytic molecules (e.g., sodium thiosulfate, urea, and heparin) (Ilic et al., 1999; Zewert et al., 1999).

The major barrier to molecular transport of the skin is its uppermost stratum, the stratum corneum (SC), which consists of ~20 layers of flattened, enucleate, keratin-filled corneocytes surrounded by lamellae of ~8 lipid bilayers on average. The lipid bilayers consist primarily of cholesterol, free fatty acids, sphingolipids, and ceramides, most of which have saturated fatty acids (Swartzendruber et al., 1989). We found that these lipid multilayers were significantly disrupted as a result of electroporation. Using time-resolved freeze-fracture electron microscopy, we observed

Submitted January 25, 2002, and accepted for publication June 4, 2002.

Address reprint requests to Dr. Sek Wen Hui, Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Tel.: 716-845-8595; Fax: 716-845-8899; E-mail: sekwen.hui@roswellpark.org.

© 2002 by the Biophysical Society

0006-3495/02/10/2064/10 $2.00

the development of local disruption areas of the lipid lamellae in the SC, in the form of vesicle formation (Gallo et al., 1999, 2002). Structural disruption was also detected by FTIR as increased SC hydration (Takeuchi et al., 2000), and by small angle x-ray diffraction (SAXD) as increased structural disorder (Jadoul et al., 1998b, 1997). Because SC lipids are involved in structural alteration by the electric pulses, we expect that modifying the SC lipids will have an effect on transdermal transport by electroporation. The use of lipids as transport enhancers promises the opportunity for transport enhancement with less structural disruption than other enhancers. We applied negative pulses to the epidermis in the presence of anionic phospholipids mixed with the marker molecules to be transported. We expected that co-transportation of anionic phospholipids with marker molecules into the epidermis would modify the barrier characteristics of the epidermis.

MATERIALS AND METHODS

Lipids and other chemicals

Lipids

Dimyristoylphosphatidylserine (DMPS), phosphatidylserine from bovine brain (brain-PS), dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidylglycerol (DOPG) were obtained from Avanti Polar Lipids (Birmingham, AL). Lipid dispersions were prepared by drying the lipids from chloroform solutions, then vortexing in Tris buffer (Tris, pH 8.0, 150 mM NaCl) at a final lipid concentration of 0.2–5 mg/ml. Anionic phospholipids form small oligolamellar vesicles under this condition.

Fluorescence molecules

Carboxyl fluorescein (CF), rhodamine-labeled phosphatidylethanolamine (rhodamine-PE), and FITC-labeled Dextran (MW 4000 and 9000) were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of epidermis

Porcine full-thickness belly skin was obtained fresh from a local abattoir. Pieces of the skin were heated to 60°C for 2 min, and the epidermis was gently peeled off from the skin. The fresh epidermis was placed on glass microscope slides and kept dry at 4°C until used. Before use the epidermis was placed in a dish of distilled water and hydrated for an hour. A fresh sample was used for each experimental run.

Measurement of electrical resistance

The electric impedance of the epidermis was measured using a vertical diffusion holder (Crown Glass Company, Somerville, NJ). Hydrated epidermis was placed between the two chambers, which were equipped with electrodes and filled with Tris buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0). The electrodes were made of 0.25-mm-diameter platinum wires, forming concentric rings that were 1.6 mm away from and facing the epidermis sample. The electrodes were flame-cleaned after each experimental run. The electric impedance of the SC before, during, and after the applied pulse was measured as described previously (Gallo et al., 1997, 2002). Briefly, a pulse generator and the alternating current source were connected to a voltage divider, containing a 2.2-kOhm load resistor (R_L) and the epidermis in series. The low voltage source was used to measure pre-pulse and post-pulse resistance. The pulse signal (high voltage) was used to permeabilize (for ion conductance) the epidermis and to measure its resistance during the pulse. The overall voltage across the R_L and the epidermis, V_o, and the voltage across the epidermis, V_s, were registered by a recording oscilloscope. The capacitance of the epidermis was assessed by the rising-decaying time of V_s in response to a square pulse V_o. The capacitive part of the epidermis impedance was determined to be insignificant in comparison with its resistive part. The resistance of the epidermis, R_s, was then computed as:

$$R_s = R_L V_s/(V_o - V_s)$$

The error in these resistance measurements was assessed using a standard 22-kOhm resistor in place of the epidermis. The residual resistance of the chamber, consisting of the resistance of the electrodes and buffer solution, was measured with the epidermis deliberately perforated by needles after the experiment. The residual resistance was typically 1–2 kOhm, contributing to ~10% of the epidermis resistance before electroporation.

Measurement of transport of test molecules

Porcine epidermis was placed between the two chambers of a vertical diffusion holder (Crown Glass Co., Somerville, NJ). Both upper (donor) and lower (receiver) chambers were filled with Tris buffer. The test molecules and the lipid dispersion were added to the upper (donor) chamber. Platinum wire electrodes were placed close to the epidermis on both the donor and the acceptor sides. Electroporation was carried out with a pulse generator (model 345, Velonex, Santa Clara, CA) delivering multiple square pulses up to 250 V, at a frequency of 1 Hz with a 1-ms pulse width. Transport of the small molecule CF was determined by removing aliquots from the lower (receiver) chamber. The transport of the FITC-Dextrans (MW 4000 and 9000) was determined by removing the total content of the lower chamber and concentrating under vacuum to 2.5 ml. The total amount transported for all the molecules tested was determined by measuring the fluorescence intensity using calibration curves prepared with known amounts of the test molecules.

Measurement of retention and transport of lipids

Rhodamine-PE was used as a tracer to measure the amount of exogenous lipids incorporated in the epidermis versus that passing through the epidermis into the receiver chamber. DMPS, DOPS, and DOPG were used. Direct fluorescence labeling of these phospholipids would result in altering their headgroups or acyl chains, hence affecting their interaction with SC lipids. In this study, 0.4 mol % of saturated rhodamine-DPPE or the unsaturated rhodamine-DOPE was mixed with, respectively, saturated and unsaturated anionic phospholipids during liposome formation. Assuming the extent of de-mixing of labeled phospholipids and the exogenous anionic phospholipids is minimal during incorporation in and transport through the epidermis, the distribution of fluorescence intensity should indicate the distribution of the respective exogenous phospholipids. The anionic liposomes (5 mg/ml) with respective saturated or unsaturated rhodamine-PE label were placed in the donor chamber, and the epidermis was subjected to 60 pulses of −140 V and 1 ms duration at 1 Hz. The total fluorescence of the washed and solubilized epidermis (in Solvable) and that of the fluid in the receiver chamber were measured.

Microscopic observation of localization of exogenous anionic phospholipids

Rhodamine-PE was used as a tracer to determine the location of exogenous phospholipids incorporated in the SC. Again, DMPS, DOPS, and DOPG

were used. The water-soluble small molecule CF as well as FITC-Dextran (MW 4000) were used as the test molecules for transport. Buffer solutions containing 20 μg/ml CF or 6 mg/ml FITC-Dextran-4k, and 5 mg/ml DMPS liposomes labeled with rhodamine-PE, were placed in the donor chamber. Sixty negative electric pulses of 140–250 V and 1 ms duration at 1 Hz were applied to the donor chamber side of the epidermis. The epidermis was removed and washed 10 times after pulsing and observed by conventional and confocal fluorescence microscopy. Conventional fluorescence micrographs were taken using a SPOT digital camera attached to an Olympus IMT-2 microscope with a ×10 objective. The depth profiles of CF- and rhodamine-labeled phospholipids in 2-μm-thick optical sections of the epidermis were examined using a BioRad 600 confocal microscope with a ×20 Nikon objective. The projected top view (*x-y* plane) and the depth profile (cumulative *x-z* projections) were reconstructed with the BioRad 600 software.

RESULTS

To test the importance of lipid acyl chain saturation and headgroups in lipid-enhanced transport and the incorporation of lipid enhancers in the SC, we used DMPS, brain-PS, DOPS, and DOPG as exogenous phospholipids. We measured and compared the electric-pulse-induced transport of fluorescein-labeled Dextrans (4000 and 9000 MW) in the presence and the absence of liposomes made of these anionic phospholipids (at 0–5 mg/ml lipid).

Measurements of electrical resistance breakdown and recovery

The effect of anionic phospholipids on permeabilization of the epidermis by electric pulses was measured as the extent of electric resistance decrease during applied pulses and the rate of resistance recovery after pulses. For this experiment, 30 pulses at 140 V for 1 ms and at 1 Hz were applied across the epidermis between two chambers. The lowering of resistance during the pulse and the recovery of resistance after the pulse were compared using different anionic phospholipids, and different amount of phospholipids in the donor chamber.

During pulses at this voltage, the resistance was lowered to a value close to the residue resistance of the sample holder, i.e., as measured with the epidermis samples perforated mechanically by a needle. This holds true with or without anionic phospholipids. Initially, the resistance recovered to a pre-pulsed value within milliseconds. However, recovery became slower after multiple pulses. The recovery after 30 pulses was monitored for 10 min, as depicted in Fig. 1. Here, the recovery is plotted as ratios to the value measured immediately after the cessation of pulse application. The normalization is necessary for comparison purposes because pieces of epidermis differ from each other in their pre-pulse and post-pulse resistance due to sample-to-sample variation. Yet, for the same treatment, their recovery rates are consistent.

Fig. 1 shows that the recovery rates are linear within the time range studied. The best-fit recovery slopes are dependent on the nature of anionic lipids as well as on the amount of lipids added to the donor chamber. At 1 mg/ml, unsaturated DOPS and DOPG had negligible effect on recovery, whereas saturated DMPS at this concentration and higher significantly impeded resistance recovery. The effect of DMPS was concentration dependent, as shown in Fig. 2. The effect seemed to reach saturation at a concentration of 1 mg/ml. The recovery slopes of brain-PS, with partially saturated acyl chains, were greater (steeper) than that of DMPS, as expected, and showed a similar lipid-concentration-dependent effect (Fig. 2).

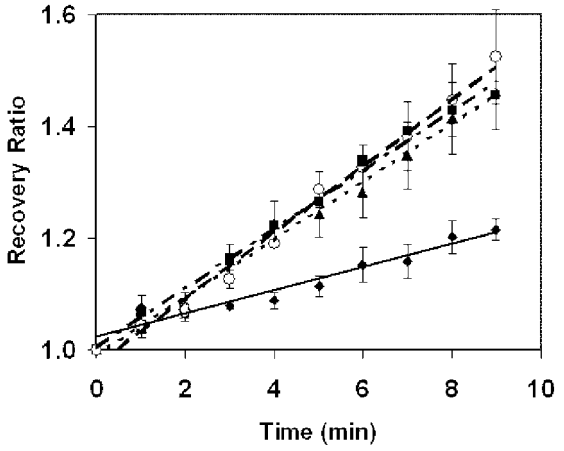

FIGURE 1 Recovery of electrical resistance of the epidermis in ratio to the resistance measured immediately after the cessation of a train of 30 pulses at 140 V for 1 ms at 1 Hz, in the presence or absence of anionic lipids in the donor chamber. ■, DOPG; ▲, DOPS; ♦, DMPS; ○, no lipid. Lines are results of linear best fit. Error bars represent variations between results from three repeated experiments.

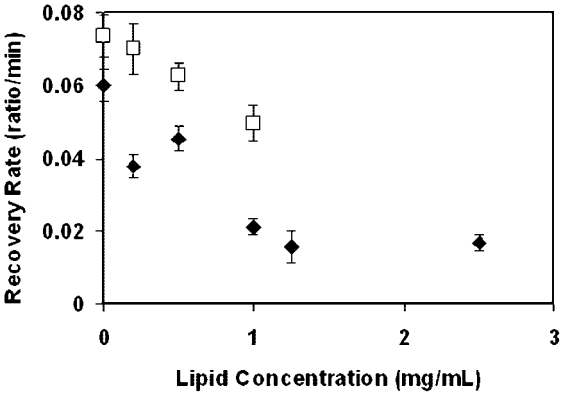

FIGURE 2 The rate of resistant recovery, as derived from the slopes of lines similar to that shown in Fig. 1, are plotted as functions of anionic lipid concentrations. ♦, DMPS; □, brain-PS. Error bars represent linear fitting errors of the mean, without taking into account the variations of repeated experiments, which are in the same order as those shown in Fig. 1.

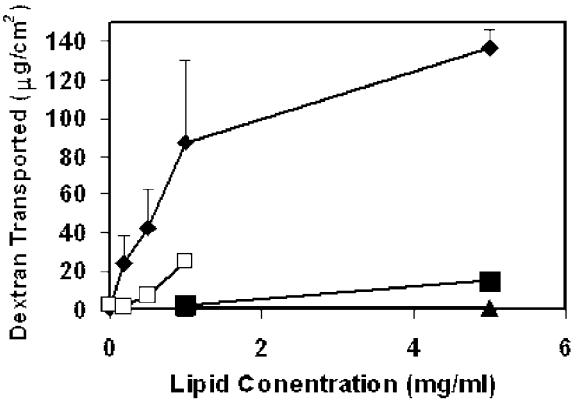

FIGURE 3 The transport of FITC-Dextran-4k across the epidermis after 30 pulses at 140 V for 1 ms at 1 Hz, in the presence of various concentrations of anionic lipids in the donor chamber. ♦, DMPS; □, brain PS; ▲, DOPS; ■, DOPG. Error bars represent variations between results from three repeated experiments.

Enhancement of transport by exogenous anionic phospholipids

All studied anionic phospholipids weakly enhanced the transport of small, water-soluble molecules, such as protoporphyrin IX (Sen et al., 2002). The enhancement ratio was ~2, and is similar for DMPS, DOPS, and DOPG.

Differential enhancement was observed with larger molecules and lower charge-to-mass ratios. Fig. 3 shows that the Dextran-4k transport increased with increasing concentration of anionic phospholipids in the donor chamber. The enhancement of Dextran-4k transport was much greater with added saturated DMPS as compared with the unsaturated DOPS, whereas brain-PS, with a mixture of acyl chain length and saturation, was in between. There was a slight difference between the effects of unsaturated DOPS and DOPG. The error bars represent standard deviations of results from at least three experiments. Variation between epidermis samples accounted for most of the data dispersion. The enhancement ratios of 1 mg/ml DMPS, brain-PS, and DOPS versus control (at zero lipid concentration), were ~80, 12, and 1, respectively. The relative efficiency in transport enhancement of saturated and unsaturated exogenous phospholipids indicates that saturation of acyl chains was the primary determinant of enhancement efficiency. The comparison between DOPG and DOPS indicates that anionic phospholipid headgroup specificity may be secondary.

Transport of Dextran-9k was much lower than that of Dextran-4k, in agreement with previous findings using DOPG/DOPC mixtures (Sen et al., 2002). Under the same conditions as Dextran-4k transport measurements, the transport of Dextran-9k in the presence of 1 mg/ml DMPS or DOPS was 11 or 5 μg/cm^2, respectively (versus 87 μg/cm^2 of Dextran-4k with DMPS). However, because the transport of Dextran-9k in the absence of anionic lipid was very low

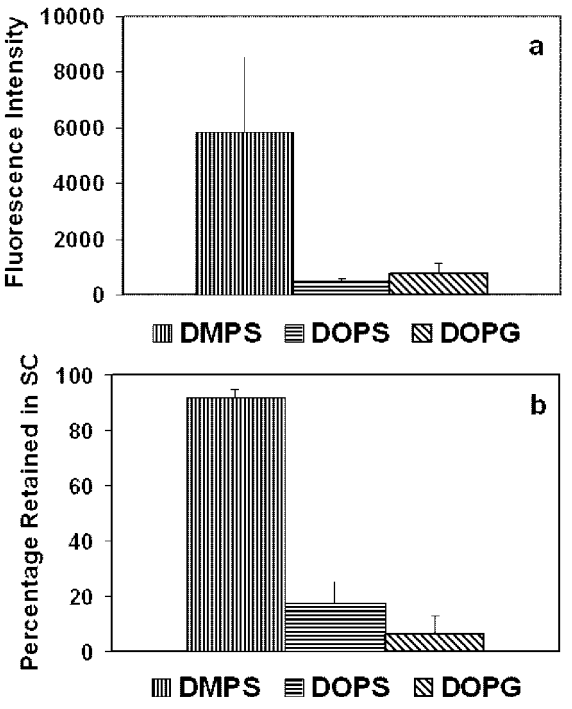

FIGURE 4 The intensity (*a*) and percentage (*b*) of fluorescence of rhodamine-labeled lipids retained in the epidermis after 30 pulses at 140 V for 1 ms at 1 Hz, in the presence of DMPS (*vertical lines*), DOPS (*horizontal lines*), and DOPG (*diagonal lines*). Error bars represent standard deviations of three repeated experiments.

(125 ng/cm^2), the enhancement ratios of DMPS and DOPS were ~90 and 40, respectively.

Partition of exogenous anionic phospholipids in the SC

Fig. 4, *a* and *b*, represent, respectively, the amount and percentage retention of fluorescence in the epidermis, after pulsing with DMPS, DOPS, or DOPG containing 0.4 mol % rhodamine-PE in the donor chamber. The error bars represent standard deviations of results from at least three experiments. If all anionic phospholipids were assumed to be associated with the labeled rhodamine-PE, then the amounts of anionic phospholipid incorporated in the epidermis after pulsing, as calculated from the fluorescence intensity in Fig. 4 *a*, were 5.4 ± 3.1, 0.45 ± 0.08, and 0.71 ± 0.36 μg/cm^2, respectively, for DMPS, DOPS, and DOPG. Apparently, DMPS was retained in the epidermis much more than the other two. Under the applied pulsing conditions, the majority of DMPS was retained by the epidermis, whereas most of the DOPS and DOPG were transported through the epidermis to the receiving chamber (Fig. 4 *b*). The results indicate that more anionic phospholipids with saturated chains were retained in the SC, regardless of headgroups.

Localization of exogenous anionic phospholipids in pulsed epidermis

The aim of this experiment was to determine whether the exogenous anionic phospholipids were localized in discrete areas of the epidermis and whether these areas were related to the transport route. The water-soluble CF, as well as FITC-labeled Dextran-4k and Dextran-9k, were used as molecules to be transported. These negatively charged molecules have been shown to pass through porcine epidermis with negative electric pulses, and the transport was enhanced by the presence of anionic phospholipids (see previous sections). Their green fluorescence indicates the hydrophilic channels and space accessible by water-soluble substances during the transport process. We used liposomes made either of DMPS with 0.4 mol % rhodamine-DPPE or of DOPS with 0.4 mol % rhodamine-DOPE to trace the location of exogenous phospholipids by fluorescence microscopy. The distribution of the red rhodamine fluorescence intensity indicates the distribution of the exogenous anionic phospholipids.

Control epidermis samples (mounted in a holder and exposed to CF or Dextran, with or without liposomes, for 1 min but not pulsed) were mostly dark when observed in a fluorescence microscope. Only faint yellowish green autofluorescence of the epidermis, with very weak, irregular shades of unwashed fluorescein, and rare spots of rhodamine fluorescence from liposomes, remained on the epidermis surface (micrograph not shown). When only CF (or Dextran) and no rhodamine labels were used, samples subjected to 140-V pulses showed individual as well as clusters of distinctly bright green fluorescent discs ~0.05–0.1 mm in diameter. These discs indicate CF remaining in the epidermis after pulsing and washing. Certain discs showed hollow centers. The percentage of hollow discs, or rings, increased with increasing pulse voltage. When no liposomes were used, the discs were much smaller, and rings did not appear unless very high voltage (250 V) pulses were applied. The observed discs were similar to those reported by Prausnitz et al. (1996), using calcein as the molecule to be transported. These discs were referred to as local transport regions (LTRs) (Pliquett et al., 1996).

If rhodamine-labeled liposomes were present in the donor chamber, many of the green fluorescent discs were observed with red dots in them, indicating the location of rhodamine-labeled phospholipids. The red dots were always associated with the green discs. An example micrograph of the epidermis after 60 pulses at 140 V, in the presence of DMPS liposomes, is shown in Fig. 5, *A* and *B*, using the green and red fluorescence filter clusters, respectively. The fluorescence of FITC-labeled Dextran-4k and rhodamine-labeled lipids was considerably brighter than the yellowish autofluorescence of the epidermis in the background.

When higher voltage pulses (≥200 V) were applied to the epidermis under otherwise the same conditions, larger, green but hollow rings (LTRs) became the dominant feature in pulsed epidermis. If rhodamine-labeled PE were also used to locate the exogenous phospholipids, the dark core regions were seen with bright red markings, indicating that exogenous phospholipids were present in the core volume of the LTRs (Fig. 5, *C* and *D*). If the micrograph were taken with a green fluorescence filter cluster, plus a dim bright-field background, corneocytes could be discerned in addition to the green discs (Fig. 5 *E*). These discs, typically 50 μm in diameter, seemed to preferentially, but not exclusively, appear along the boundaries between corneocytes, which were at least twice the width of the discs. A micrograph of lower magnification is shown in Fig. 5 *F* to illustrate the distribution of discs over a wider field. Analogous micrographs of samples pulsed in the presence of DOPS instead of DMPS are shown in Fig. 6, *A–F*. Although the numbers of green discs were similar in both DOPS and DMPS samples, there was less red fluorescence in DOPS samples, indicating fewer DOPS molecules associated with the epidermis under identical pulsing condition as for DMPS samples. Within the LTRs, red fluorescent lipids usually occupied only the center portion of the LTRs in DOPS samples, whereas in DMPS samples, the entire LTRs were infiltrated by red fluorescent lipids (Figs. 5 and 6).

The number of green discs in each epidermis sample was counted from the microscope images. The sampling area spanned ~75% of the total areas of each epidermis sample. For the same number of pulses, the density of discs increased with pulse voltage (Fig. 7). The error bars represent variations among different sampling areas. There was no significant difference in disc densities between epidermis samples pulsed in the presence of DMPS or DOPS.

When CF was used in place of Dextran, the disc morphology appeared similar, except brighter and larger. A typical micrograph of the epidermis after 60 pulses at 250 V, taken with a ×30 objective, is shown in Fig. 8 *c*. Because of the uneven surface of the epidermis, some areas of the field are out of focus. Confocal microscopy was applied to determine the depth profile of the exogenous phospholipid with respect to the green fluorescent volume of CF penetration. The projected top view (*x-y* plane) and the depth profile (cumulative *x-z* projections), reconstructed from 36 of the 2-μm-thick *x-y* layer images, of a single green disc are shown in Fig. 8, *a* and *b*, respectively. The vertical span of the image in Fig. 8 *b* is thus 72 μm (*x* and *z* scales different). It shows that, whereas the CF discs spread wide laterally, the red fluorescence representing rhodamine-labeled lipids remain at the center portion of the CF disc by the electric pulses.

DISCUSSION

Although the skin poses a formidable barrier to transport of most substances, especially water-soluble materials, methods have been devised to overcome this barrier property.

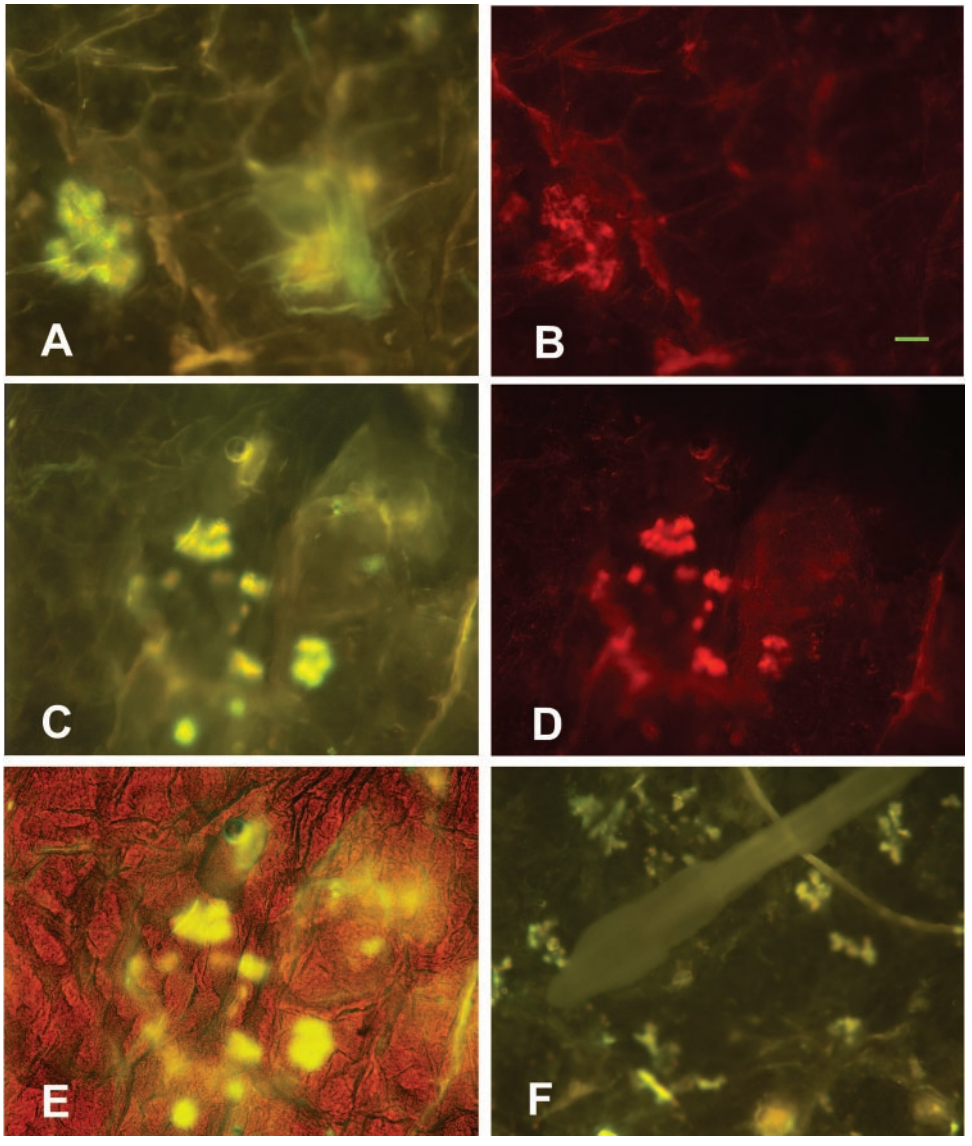

FIGURE 5 Fluorescence micrographs of the epidermis after 60 pulses for 1 ms at 1 Hz, at a pulse voltage of 140 V (*A* and *B*) or 200 V (*C–F*). The donor chamber contained FITC-Dextran-4k and rhodamine-labeled lipids (DMPS). The pair *A* and *B*, and the triplet *C*, *D*, and *E*, are of the same fields, respectively. (*A*, *C*, and *F*) Recorded with a green (fluorescein) filter; (*B* and *D*) Recorded with a red (rhodamine) filter cluster. Bright green regions (discs) in *A*, *C*, and *F* indicate the presence of FITC-Dextran-4k in the SC, whereas bright red regions in *B* and *D* and the under-filtered red in *A*, *C*, and *F* in the centers of green discs indicate the presence of rhodamine-labeled lipids in the SC. (*E*) Recorded with a green filter cluster plus a bright-field (orange) background lighting to illustrate corneocyte boundaries (with a green tint of FITC-Dextran-4k; (F) Recorded with a lower-magnification objective. This field contains a piece of hair. The scale bar in B is 100 μm for *A–E* and 250 μm for *F*.

However, methods developed so far, such as chemical transport enhancers and iontophoresis, apply only to small molecules of $<$1000 Da. The main transport route is through the appendages. Electroporation, on the other hand, creates a direct pathway through the SC, by-passing the appendages (Pliquett et al., 1996). The upper molecular size limit of transdermal delivery by electroporation is still $<$10 kDa. Increasing the upper limit of the molecular size is desirable because many drugs and biological modifiers, including insulin, are still beyond this limit. Attempts have been made to increase the upper limit by applying keratolytic molecules (e.g., sodium thiosulfate, urea, and heparin) (Ilic et al., 1999; Zewert et al., 1999) simultaneously with electroporation, to enlarge the transport channels. The idea is to cause more and longer-lasting transportation routes by the applied pulses. Our approach here is to use exogenous anionic lipids for this purpose.

SC lipids consist mainly of sphingolipids and ceramides, most of which have saturated fatty acids (Swartzendruber et al., 1989). We found that adding cationic lipids, such as dioleoyl-3-trimethylammonium propane (DOTAP), to the milieu of molecules for transdermal transport by electropora-

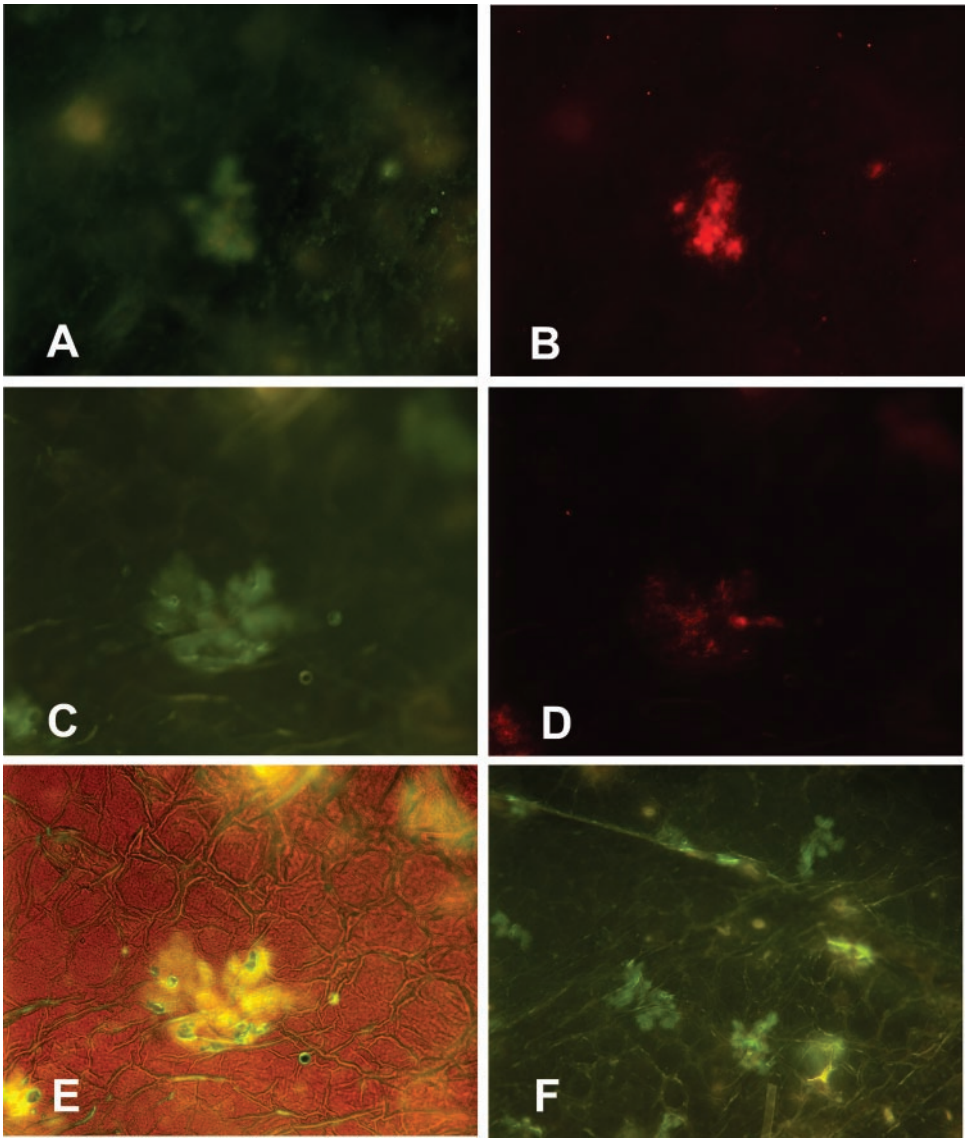

FIGURE 6 Analogous fluorescence micrographs of the epidermis as in Fig. 5, except that the donor chamber contained FITC-Dextran-4k and rhodamine-labeled lipids (DOPS).

tion inhibited transport efficiency (unpublished results). We believed that cationic lipids would bind to the surface of the skin, which, in general, has a negative charge. These additional layers would make creating transport routes by electroporation more difficult, impeding transport. Adding neutral lipids to the milieu had no effect on transdermal transport by electroporation (unpublished results). Only anionic lipids produced transport-enhancing effects. The enhancement ratio can be as high as two orders of magnitude (Fig. 3) (Sen et al., 2002). Our rationale is that, because of their net negative charge, anionic lipids could be driven into the SC by negative pulses. (Indeed, applying pulses of reversed polarity did not result in transport enhancement). From our electron microscopy studies (Gallo et al., 1999, 2002), we found that, upon electric pulse application, tightly

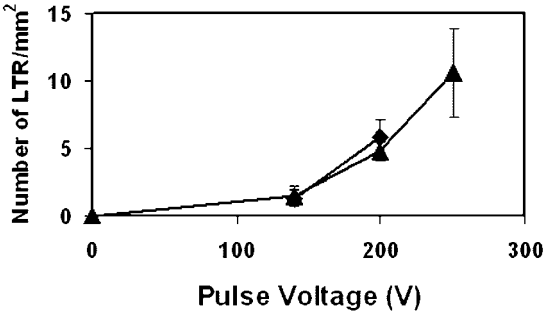

FIGURE 7 The density of LTRs as functions of applied pulse voltage, in the presence of DMPS (♦) or DOPS (▲). LTRs were counted based on the number of green discs within a given area, from micrographs similar to Figs. 5 *F* and 6 *F*. Error bars represent standard deviations of data derived from at least three micrographs.

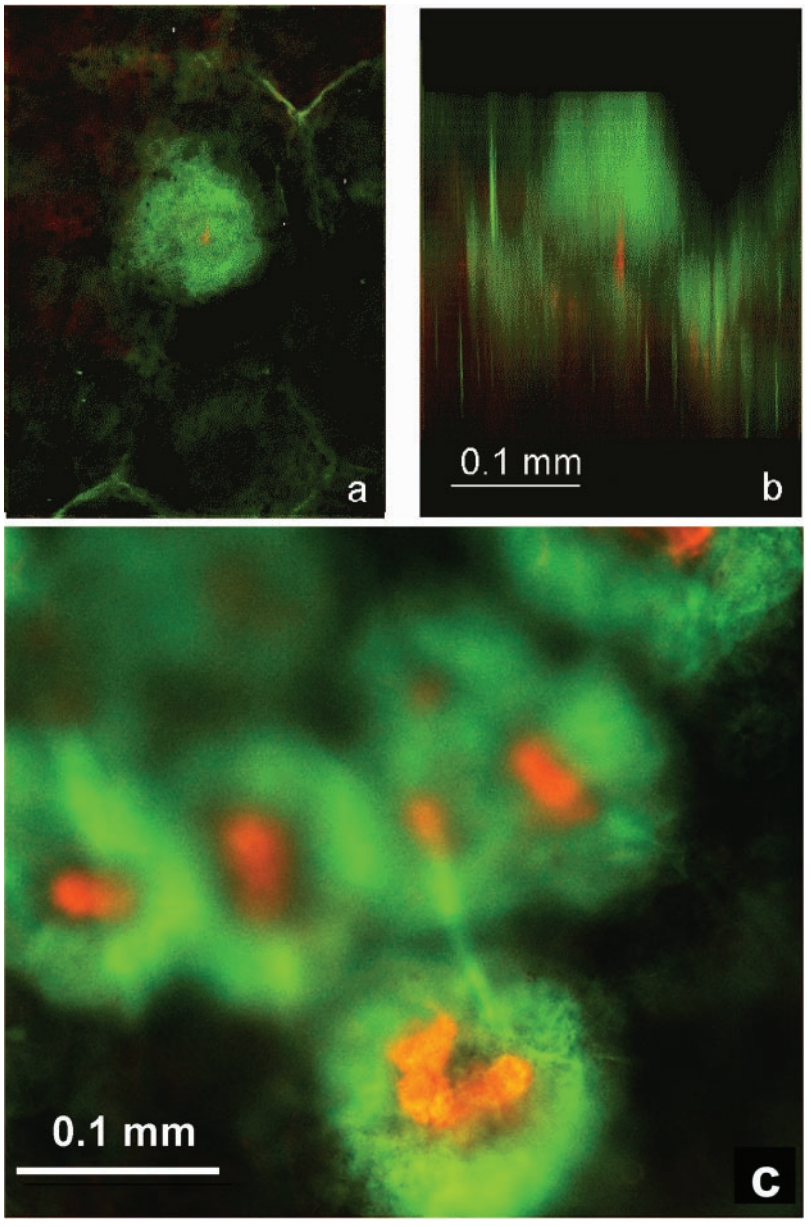

FIGURE 8 Conventional (*c*) and confocal (*a* and *b*) fluorescence micrographs of the epidermis after 60 pulses for 1 ms at 1 Hz, at a pulse voltage of 250 V. The donor chamber contained CF and rhodamine-labeled lipids (DMPS). Green fluorescent discs represent CF-containing LTRs. In the centers of LTRs, red rhodamine-labeled lipids can be seen. The micrograph (*c*) was recorded with a green fluorescence filter cluster with sufficient red pass. The top-view confocal micrograph (*a*) was reconstructed by superimposing 36 (*x-y*) layer images, each 2 μm thick. Green CF was seen within the LTR disc and along corneocyte boundaries. The side-view (*x-z*) confocal micrograph of the same field (*b*) was reconstructed from the same layer images. The vertical span is thus 72 μm thick (*z*-scale is approximately three times the *x-y* scale).

packed lipid lamellae between corneocytes in the SC were transformed into vesicular patches that eventually reverted back to lamellae after seconds to minutes. Columns of these patches or aggregates though the SC are thought to be a form of passageway for transdermal transport created by electroporation (Gallo et al., 1999). Because anionic lipids prefer loosely packed multilamellae (Stewart et al., 1979) and are prone to develop and retain vesicular form upon physical perturbation, such as electric pulses, we expect that the presence of anionic lipids in SC would lower the breakdown threshold voltage and/or prolong the recovery. Enhancing and prolonging the vesicle column formation during and after electroporation is likely to be the basis of transport enhancement by anionic lipids.

The fate of exogenous lipids in the SC is an important consideration. The gel-to-fluid phase transition of SC lipids is ~70°C. It is believed that local heating by the electric breakdown triggered by the applied pulse induces local melting of the SC lipid lamellae and leads to vesicle formation (Pliquett et al., 1996; Gallo et al., 2002). Endogenous SC lipid vesicles revert back to tightly packed planar lamellae form at room temperature after the cessation of pulses (Gallo et al., 1999, 2002), by spontaneous fusion, as expected for long-chain, low-hydration, saturated lipids (Marsh, 1990; Seddon, 1990). Exogenous lipids with similar hydration and saturation are expected to behave likewise and are more likely to fuse with SC lipids into the SC lamellae. Incorporation of anionic lipids into the SC lipid

lamellae is expected to disrupt the tightly packed SC lipid lamellae. Exogenous lipids with unsaturated chains remain in the fluid phase at room temperature and are less likely to form planar lamellae under similar dehydration conditions. Therefore, their fusion and mixing with SC lipids are less likely. The phenomenon is different from cell-liposome fusion, which does not usually proceed spontaneously because of the highly hydrated and more fluid nature of cell membranes (Stromberg et al., 2000). SC lipids have much lower hydration, and the dried corneocytes membranes are very different from living cell membranes.

The fluorescence microscopic observation of this study supports this hypothesis. Anionic phospholipids were seen to penetrate into the epidermis and were found mainly in the SC lipid lamellae or situated in the centers of the transport regions marked by CF or FITC-Dextran. It is likely that the anionic phospholipids were driven into the SC at various focal spots where electric breakdown occurred along the least resistive paths, and narrow local conductive channels were created initially. Some anionic phospholipids remained in the SC and could become constituents of columns of vesicles as the channels widen with the energy deposited by the pulse current. Water-soluble fluorescent markers, such as CF and FITC-Dextran, would pass through as well as migrate laterally into epidermis areas surrounding the initial channels, forming the visible LTR. The absence of these marker molecules in the center of the ring-shaped LTR observed after high voltage and prolonged pulses indicates that these marker molecules have been driven through to the receiver chamber, leaving the outlying molecules forming the rings. If labeled lipids were present, they were seen throughout the LTR but more prominently in the centers of the LTR. The dimension of vesicle aggregates in SC pulsed under similar conditions and observed by freeze-fracture electron microscopy (1–10 μm in diameter) (Gallo et al., 1999) are similar to the LTRs we observed by fluorescence microscopy (Figs. 5–8). The fractional area covered by vesicle aggregates (0.05–0.5% of the total sampled area) are less than that by LTR (Figs. 5–8), because of lower pulse voltage (80 V), a fewer number of pulses (1), and lower probability of sampling (a single plane within the SC) in the study by freeze-fracture electron microscopy (Gallo et al., 1999).

Among the anionic phospholipids we tested, the effects of headgroup specificity on resistance recovery and Dextran transport are minimal, as seen by the comparison of DOPS and DOPG (Figs. 1 and 3). Given that both headgroups have a single negative charge each, and the ability of being driven into the SC is determined mainly by the electrophoretic force, the weak influence of headgroup selectivity is not surprising. The most influential factor on resistance recovery and Dextran transport is acyl chain saturation (Figs. 1 and 3). The saturated DMPS is much more potent than unsaturated DOPS (and DOPG), and the effect is dose dependent (Figs. 2 and 3). The potency in impeding resistance recovery and enhancing Dextran transport are related to the amount of anionic phospholipids retained in the epidermis (Figs. 4–6). Under the pulse condition tested (140 V for 1 ms at 1 Hz; 30 pulses), most of the DMPS, but little of the DOPS and DOPG, are retained in the epidermis (Fig. 4 *b*). This observation supports our hypothesis that the anionic lipids retained in the epidermis helps to maintain the transport route created by electroporation.

We can envision three scenarios in which anionic lipids in the epidermis help to maintain low epidermis resistance and enhance transport: 1) anionic lipids facilitate larger transport pathways to be created by electroporation; 2) anionic lipids facilitate more transport pathways to be created by electroporation; and 3) anionic lipids prolong the opening of the pathways. The vastly different transport enhancement in DMPS and DOPS samples (Fig. 3) but nearly identical counts of LTR (Fig. 7) all but rule out the second scenario. The fact that the molecular mass cutoff is similar for DMPS, DOPS, and DOPG samples (10 kDa) (Sen et al., 2002) as well as in samples without lipid enhancers (Lombry et al., 2000) indicates that path size enlargement by anionic lipids (scenario 1) is not a significant factor. A significant retardation of resistance recovery by DMPS in a dose-dependent manner (Figs. 1 and 2) suggests scenario 3 as the most likely mechanism. A longer opening state also facilitates the transport of neutral and positively charged molecules (with negative pulses) (Sen et al., 2002); the major mechanism of transporting these molecules is by diffusion through pathways generated by electroporation. The major factor for transporting small charged molecules and molecules with high charge/mass ratio, such as CF and protoporphyrin IX, through the epidermis is electrophoresis (iontophoresis) during the pulse. Prolonged lifetime of transport pathways by saturated anionic lipids thus have less effect on the transport of these molecules. On the other hand, transport of large, neutral, or low-charge/mass ratio molecules, such as FITC-Dextran, is mainly by diffusion through pathways opened by electroporation. Prolonged opening of these pathways by saturated anionic lipids thus makes a significant contribution to the transport.

Although we have not exhaustively tested all available lipids as transport enhancers, nor do we claim that the pulsing conditions we used are optimal for transdermal transport, we are reasonably certain that the introduction of anionic lipids in the SC facilitates and prolongs the transport pathways created by electroporation. By using saturated anionic lipid enhancers for transdermal transport by electroporation, we are able to transport 4-kDa molecules at an equivalent flux of 275 μg/cm^2/min, and 9-kDa molecules at $\sim$10% of this rate. Peptide drugs, metabolic regulators such as insulin, and vaccines as well as antisense polynucleotides of these sizes can now be transported through the skin at practical rates by electroporation, without long-lasting skin damage.

This work was supported by the grant GM 55864 (to S.W.H.) from the National Institutes of Health. The vertical diffusion chambers and perfusion platform were on loan from the Department of Pharmaceutics, SUNY at Buffalo. Confocal microscopy was performed with the help of Mr. Edward Hurley of the Cell Analysis Facility, which is supported by the CCSG grant CA 16056 from the National Cancer Institute.

REFERENCES

Chen, T., E. M. Segall, R. Langer, and J. C. Weaver. 1998. Skin electroporation: rapid measurements of the transdermal voltage and flux of four fluorescent molecules show a transition to large fluxes near 50 V. *J. Pharm. Sci.* 87:1368–1374.

Clarys, P., K. Alewaeters, A. Jadoul, A. Barel, R. O. Manadas, and V. Preat. 1998. In vitro percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *Eur. J. Pharm. Biopharm.* 46:279–283.

Gallo, S. A., A. R. Oseroff, P. G. Johnson, and S. W. Hui. 1997. Characterization of electric pulse induced permeabilization of porcine skin using surface electrodes. *Biophys. J.* 72:2805–2811.

Gallo, S. A., A. Sen, M. L. Hensen, and S. W. Hui. 1999. Time-dependent ultrastructural changes in porcine stratum corneum following an electric pulse. *Biophys. J.* 76:2824–2832.

Gallo, S. A., A. Sen, M. L. Hensaen, and S. W. Hui. 2002. Temperature-dependent electrical and ultrastructural characterization of porcine skin upon electroporation. *Biophys. J.* 82:109–119.

Ilic, L., T. R. Gowrishankar, T. E. Vaughan, T. O. Herndon, and J. C. Weaver. 1999. Spatially constrained skin electroporation with sodium thiosulfate and urea creates transdermal microconduits. *J. Control. Release.* 61:185–202.

Jadoul, A., N. Lecouturier, J. Mesens, W. Caers, and V. Preat. 1998a. Transdermal alniditan delivery by skin electroporation. *J. Control. Release.* 54:265–272.

Jadoul, A., and V. Preat. 1997. Electrically enhanced transdermal delivery of domperidone. *Int. J. Pharm.* 154:229–234.

Jadoul, A., H. Tanojo, V. Preat, J. A. Bouwstra, F. Spies, and H. E. Bodde. 1998b. Electroperturbation of human stratum corneum fine structure by high voltage pulses: a freeze-fracture electron microscopy and differential thermal analysis study. *J. Invest. Dermatol.* Symposium Proceeding. 3:153–158.

Johnson, P. G., S. A. Gallo, A. R. Oseroff, and S. W. Hui. 1998. Transdermal transport of methylene blue through full-thickness porcine skin by electroporation-electrophoresis. *J. Invest. Dermatol.* 111:457–463.

Lombry, C., Dujardin, N., and Preat, V. 2000. Transdermal delivery of macromolecules using skin electroporation. *Pharm. Res.* 17:32–37.

Marsh, D. 1990. Handbook of Lipid Bilayers. CRC Press, Boca Raton, FL.

Misra, A., S. Ganga, and P. Upadhyay. 2000. Needle-free non-adjuvanted skin immunization by electroporation-enhanced transdermal delivery of diphtheria toxoid and a candidate peptide vaccine against hepatitis B. *Vaccine.* 18:517–523.

Pliquett, U. F., T. E. Zewert, and T. Chen. 1996. Imaging of fluorescent molecule and small ion transport through human stratum corneum during high voltage pulsing: localized transport regions are involved. *Biophys. Chem.* 58:185–204.

Prausnitz, M. R., V. G. Bose, R. Langer, and J. C. Weaver. 1993. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. *Proc. Natl. Acad. Sci. U.S.A.* 90:10504–10508.

Prausnitz, M. R., J. A. Gimm, R. H. Guy, R. Langer, J. C. Weaver, and C. Cullander. 1996. Imaging regions of transport across human stratum corneum during high-voltage and low-voltage exposures. *J. Pharm. Sci.* 85:1363–1370.

Regnier, V., and V. Preat. 1998. Localization of a FITC-labeled phosphorothioate oligodeoxynucleotide in the skin after topical delivery by iontophoresis and electroporation. *Pharm. Res.* 15:1596–1602.

Regnier, V., A. Tahiri, N. Andre, M. Lemaitre, T. Le Doan, and V. Preat. 2000. Electroporation-mediated delivery of 3′-protected phosphodiester oligodeoxynucleotides to the skin. *J. Control. Release.* 67:337–346.

Seddon, J. M. 1990. Structure of the inverted hexagonal phase, and non-lamellar phase transitions of lipids. *Biophys. Biochem. Acta.* 1031: 1–69.

Sen, A., Y. L. Zhao, L. Zhang, and S. W. Hui. 2002. Enhanced transdermal transport by electroporation using anionic lipids. *J. Control. Release*. In press.

Stewart, T. P., S. W. Hui, A. R. Portis, Jr., and D. Papahadjopoulos. 1979. Complex phase mixing in phosphatidylcholine and phosphatidylserine multilamellar vesicles. *Biochim. Biophys. Acta.* 556:1–16.

Stromberg, A., F. Ryttsen, D. T. Chiu, M. Davidson, P. S. Erikson, C. F. Wilson, O. Orwar, and R. N. Zare. 2000. Manipulating the genetic identity and biochemical surface properties of individual cells with electric field induced fusion. *Proc. Natl. Acad. Sci. U.S.A.* 97:7–11.

Swartzendruber, D. C., P. W. Wertz, D. J. Kitko, K. C. Madison, and D. T. Downing. 1989. Molecular models of the intercellular lipid lamellae in mammalian stratum corneum. *J. Invest. Dermatol.* 92:251–257.

Takeuchi, Y., K. Miyawaki, S. Kamiyabu, S. Fukushima, Y. Yamaoka, S. Kishimoto, K. Taguchi, H. Masai, and Y. Kamata. 2000. Use of electroporation to accelerate the skin permeability enhancing action of oleic acid. *Biol. Pharm. Bull.* 23:850–854.

Vanbever, R., G. Langers, S. Montmayeur, and V. Preat. 1998a. Transdermal delivery of fentanyl: rapid onset of analgesia using skin electroporation. *J. Control. Release.* 50:225–235.

Vanbever, R., M. A. Leroy, and V. Preat. 1998b. Transdermal permeation of neutral molecules by skin electroporation. *J. Control. Release.* 54: 243–250.

Vanbever, R., M. R. Prausnitz, and V. Preat. 1997. Macromolecules as novel transdermal transport enhancers for skin electroporation. *Pharm. Res.* 14:638–644.

Vanbever, R., and V. V. Preat. 1999. In vivo efficacy and safety of skin electroporation. *Drug Deliv. Rev.* 35:77–88.

Weaver, J. C., R. Vanbever, T. E. Vaughan, and M. R. Prausnitz. 1997. Heparin alters transdermal transport associated with electroporation. *Biochem. Biophys. Res. Commun.* 234:637–640.

Zewert, T. E., U. F. Pliquett, R. Vanbever, R. Langer, and J. C. Weaver. 1999. Creation of transdermal pathways for macromolecule transport by skin electroporation and a low toxicity, pathway-enlarging molecule. *Bioelectrochemistry.* 49:11–20.