Electroosmotic Pore Transport in Human Skin

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Purpose. To determine the pathways and origin of electroosmotic flow in human skin.

Methods. Iontophoretic transport of acetaminophen in full thickness human cadaver skin was visualized and quantified by scanning electrochemical microscopy. Electroosmotic flow in the shunt pathways of full thickness skin was compared to flow in the pores of excised stratum corneum and a synthetic membrane pore. The penetration of rhodamine 6G into pore structures was investigated by laser scanning confocal microscopy.

Results. Electroosmotic transport is observed in shunt pathways in full thickness human skin (e.g., hair follicles and sweat glands), but not in pore openings of freestanding stratum corneum. Absolute values of the diffusive and iontophoretic pore fluxes of acetaminophen in full thickness human skin are also reported. Rhodamine 6G is observed to penetrate to significant depths (~200 μm) along pore pathways.

Conclusions. Iontophoresis in human cadaver skin induces localized electroosmotic flow along pore shunt paths. Electroosmotic forces arise from the passage of current through negatively charged meso- or nanoscale pores (e.g., gap functions) within cellular regions that define the pore structure beneath the stratum corneum.

KEY WORDS: human skin; iontophoresis; electroosmosis; scanning electrochemical microscopy; confocal microscopy.

INTRODUCTION

Electroosmotic transport in human skin (1) and skin models (2) (e.g., hairless mouse) is well established and may play an important role in transdermal drug delivery (3–11). The origin of electroosmotic forces and the identification of electroosmotic flow pathways in skin, however, has received little attention in the literature. Both human skin and hairless mouse skin are negatively charged above pH 4 (1) and thus, the passage of electrical current is carried predominantly by cations. Momentum transfer from the charge carrying cations to the other solution constituents (i.e., counter ions and solvent molecules) results in solution flow in the same direction as the electrical current (12). In this report, the authors address the issue of identifying the location in skin whereby electroosmotic flow originates.

Electroosmosis in non-biologic media is frequently associated with the passage of current through charged pores that have dimensions ranging from a few nanometers to several tens of nanometers (13). Electroosmotic flow, however, is not restricted to mesoscale and nanoscale pores. In capillary electrophoresis, whereby electroosmotic flow is the basis for chemical separation, the capillary (i.e., the charged pore) radius is as large as 100,000 nm (14). Thus, in investigating electroosmosis in skin, it is necessary to consider porous structures of widely ranging dimensions as potential structures responsible for the observed flow.

Skin is a complex medium containing a variety of pore structures of different sizes (15). Large pores in skin (e.g., hair follicles and sweat glands) traverse the stratum corneum, epidermis, and dermis and are known to act as efficient shunt pathways for molecular transport (15,16). Enhanced molecular transport via electroosmosis has been demonstrated to be associated with hair follicles in full-thickness hairless mouse skin, as determined by measurement of the molecular flux at the follicle opening on the exterior side of the stratum corneum (16–19). The structures responsible for the generation of the flow in hair follicles have not been investigated. An important issue is whether flow is generated within the ~20-μm diameter hair follicle, or within the smaller porous structures within the follicle sheath. For instance, gap functions are clusters of gated intercellular channels between cells in the epidermis and dermis layers, which allow the passage of charge species in response to voltage perturbations (20). Gap junctional channels are formed by integral membrane proteins called connexins (Cx) (21). In human skin, negatively charged Cx43 is the most abundant Cx found in the epidermis and dermis layers, as well as in hair follicles and sweat glands (21).

To investigate the above issues, the authors have used scanning electrochemical microscopy (22) (SECM) to measure, *in vitro*, molecular fluxes through full thickness human skin and excised stratum corneum. The authors demonstrate that electroosmotic flow is highly localized to shunt pathways in full thickness human skin, which is analogous to previous conclusions concerning transport in hairless mouse skin (16–19). In addition, the authors show that electroosmosis is inoperative during iontophoresis across excised human stratum corneum. The results suggest that the entire shunt pathway structure is required to observe electroosmotic flow in human skin. Localized penetration of a dye molecule along significant lengths of shunt pathways in human skin is also demonstrated using laser scanning confocal microscopy.

MATERIALS AND METHODS

Chemicals

Acetaminophen (99.9%, Aldrich) and rhodamine 6G (Excitron, Inc., rhodamine 590 chloride) were used as received. Water was purified by use of a Barnstead E-pure 4-module ORGANICfree system.

Human Skin

Dermion Corporation (Salt Lake City, UT) provided the samples of full thickness human cadaver skin from the back of an 89-year-old man. The samples were kept at –37°C until used, then thawed under refrigeration between phosphate buffered saline filled (Dulbecco's) gauze pads. The skin was used within 2 h of thawing.

Human Stratum Corneum

Samples of human stratum corneum were obtained from Prof. Kris Knutson (Dept. of Pharmacology, University of

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Utah). The stratum corneum was separated from human cadaver skin by soaking the skin in trypsin (23). Samples were stored at room temperature until used.

Mica/Nafion Membrane

Synthetic cation-selective membranes containing a single pore were prepared as models of shunt pathways in human skin as previously described (18). Square samples (0.75″ × 0.75″) were cut from a 100 μm-thick sheet of mica. A circular shaped pore was created in the center of each membrane by laser ablation using the 355-nm line of a Nd:YAG pulsed laser (Spectra Physics). Five 90-mJ pluses of 10 ns duration yielded a pore with a radius ranging from 10–40 μm. To create a cation selective membrane, two ~40 μL drops of a 5% wt solution of Nafion 117 in alcohol/water (Aldrich, 1100 equivalent weight) were placed over the pore opening and drawn into the pore by capillary forces. The membrane was allowed to dry and the process was repeated on the opposite side. The resulting mica sheet containing a single pore filled with Nafion is hereafter referred as a mica/Nafion membrane.

Scanning Electrochemical Microscopy (SECM) and the Iontophoresis Cell

The SECM and iontophoresis cell used in these studies, (Fig. 1) have been previously described in detail (16–19). Briefly, the skin sample separates the receptor and donor compartments of a custom-built Teflon vertical iontophoresis cell. For increased mechanical stability, the skin samples or mica/nafion membrane were sandwiched between two glass slides, each containing a 0.5 cm^2 hole that exposed the sample to the donor and receptor solutions. Silicone vacuum grease was used to seal the membrane/glass interface. The glass Teflon interfaces were sealed using built-in 1″ diameter gaskets. Both compartments of the iontophoresis cell contain ~15 mL of an aqueous electrolyte solution (0.2 M NaCl). The donor compartment contains the permeate molecule, acetaminophen, at a concentration of 0.05 M. Localized regions of transport across the skin sample are visualized and quantified by amperometric detection of the oxidation of acetaminophen at the SECM tip, which is rastered at a height of ~15 μm above the skin surface in the receptor compartment. The potential of the SECM tip is held at ~0.40 V positive of the half-wave potential for acetaminophen oxidation, $E^{\circ} = 0.60$ V vs. Ag/AgCl, to ensure mass-transport limited oxidation of this species. The Faradic current that results from oxidation of acetaminophen at the SECM tip is proportional to the local concentration of acetaminophen, Eq. (1), (24),

$$i_t = 4nFDC(x,y,z)r_t$$

where n is the number of electrons transferred (= 1), F is Faraday's constant, and D and C(*x*,y,z) are the diffusivity (9.1 × 10^{-6} cm^2/s) (18) and local concentration of acetaminophen, respectively. The factor of 4 in Eq. (1) reflects the disk-shape geometry of the tip electrode (*vide infra*). SECM images are constructed by plotting the SECM tip current i_t as a function of lateral coordinates, *x* and y, at fixed separation distance, z = 15 μm. A high local concentration of acetaminophen above the skin sample indicates a high local acetaminophen flux through the skin. If the tip-to-skin separation distance is held constant during imaging, the variation in SECM tip current as a function of *x* and y is exactly proportional to the local flux. Thus, SECM images allow visualization of local molecular fluxes in skin.

The SECM tip position is controlled by a piezoelectric inchworm microtranslation stage (model TSE-75, Burleigh Instruments) with a precision of 0.1 μm. A low-noise custom-built potentiostat, controls the tip potential with respect to a Ag/AgCl reference electrode (16). Tip current is measured with a precision of 2 pA. In these experiments, the iontophoretic current, i_{app} is applied across the skin samples using a galvanostat (model RDE-4, Pine Instruments). Iontophoretic currents labeled as "positive" correspond to current flow (cation migration) from the donor to the receptor compartment.

Carbon fiber SECM microelectrodes (1.1–4.8 μm radius) were constructed as previously described (18). Briefly, the carbon fiber was insulated with an electropolymerized film (~250 nm) of poly(oxyphenylene). The fiber tip was then cut at the end with a sharp razor blade to expose a disk-shaped carbon electrode.

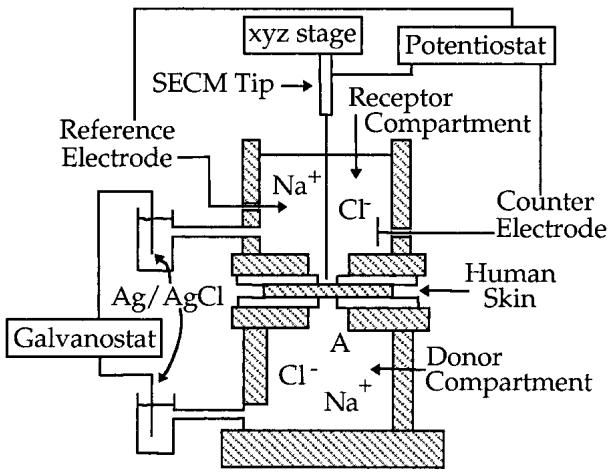

Fig. 1. Schematic diagram of the iontophoresis cell and SECM. Acetaminophen (Ac) is dissolved in the donor compartment and is transported upwards across either full-thickness human skin or human stratum corneum. The SECM tip is rastered across the sample and used to amperometrically detect acetaminophen as it emerges into the receptor compartment. The galvanostatic circuit and two large Ag/AgCl electrodes are used to apply a constant iontophoretic current across the skin sample.

Laser Scanning Confocal Microscopy

LSCM images were obtained using a LSM 510 microscope (Carl-Zeiss, Jena, Germany) with a 20× objective. A HeNe laser (543 nm) was used as the excitation source, incorporating a long pass 560 nm filter. The pinhole was set at 94 μm to obtain 4.5 μm-wide optical slices.

RESULTS AND DISCUSSION

Localized Transport in Human Skin

In previously published reports, the authors demonstrated that the diffusive and iontophoretic flux of small organic and inorganic molecules through full thickness hairless mouse skin is highly localized to hair follicles[18,17]. In this investigation, SECM images indicate that molecular transport in full-thickness human cadaver skin is also localized.

Figure 2 shows a SECM image of a 300 × 225 μm^2 region of full-thickness human skin recorded during iontophoresis of acetaminophen at an applied current, i_{app}, of 100 μA. The steady-state image is obtained by recording the Faradic current corresponding to the oxidation of acetaminophen at the SECM tip as the molecule emerges from the skin (see experimental section). The bright region at the center of the image corresponds to a local high concentration of acetaminophen. Such a highly localized concentration indicates that the rate of transport of acetaminophen from the skin into the receptor compartment is also localized. This image, which is representative of other images of full thickness human skin, suggests that the flux of acetaminophen in the skin sample is constrained within microscopic pores. Quantitative SECM measurements (*vide infra*) yield pore opening radii of ~25 μm. No attempt was made in this preliminary study to measure the number density of shunt pathways or the fraction of the total flux through the shunts. Eight separate skin samples were imaged with results similar to that as shown in Fig. 2.

The SECM image in Fig. 2, demonstrates that the flux of acetaminophen is localized as this species emerges from the skin tissues. However, SECM does not directly provide information about transport beneath the skin surface. Laser scanning confocal microscopy (LSCM) was used to obtain depth profiles of molecular species concentrations following iontophoresis (25). A sample of full thickness human skin was positioned in the iontophoresis cell and exposed for 30 min to a 2.0 mM rhodamine 6G solution containing 0.2 M NaCl during iontophoresis at 100 μA. The skin sample was then rinsed with deionized H_2O and imaged by LSCM. A series of LSCM images of a 1 × 1 mm^2 region of skin, obtained at 20-μm depth intervals with 4.5 μm width optical slices, is presented in Fig. 3. Although rhodamine 6G seems to stain keratin fibers in the upper regions of the skin, a localized circular shaped fluorescence signal is observed to depths of ~200 μm. (Loss of the fluorescence signal at greater depths is due, in part, to increasingly poorer collection efficiency. Special care was taken to ensure focus on the surface of the stratum corneum.) Numerous circular-shaped fluorescence signals (~20 per 921 × 921 μm^2 image) were observed. A feature of these images is that the lateral position of the localized fluorescence does not significantly vary with depth. This finding suggests that rhodamine 6G is transported through shunt pathways that extend from the stratum corneum to a depth >200 μm.

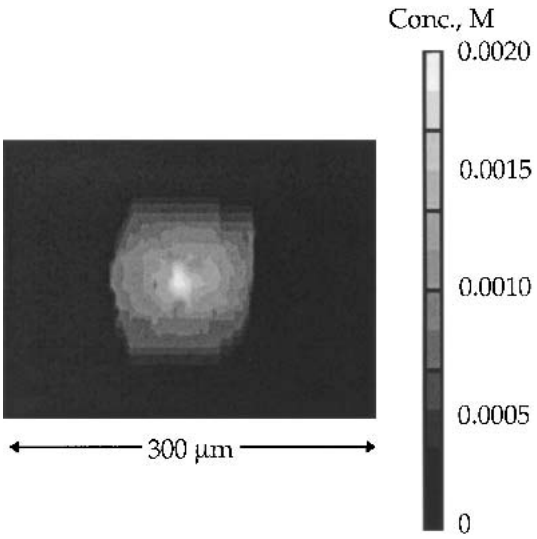

Fig. 2. SECM image of the concentration of acetaminophen above a pore opening in full thickness human skin. The image was acquired during iontophoresis with i_{app} = 100 μA. The SECM tip was rastered at a height of 15 μm above the skin, at a scan rate of 1 μm/s. The bright region corresponds to a high concentration of acetaminophen.

The SECM and LSCM images provide strong evidence that iontophoretic transport of acetaminophen in human cadaver skin is localized to porous shunt pathways. SECM provides direct evidence of large local steady-state fluxes, whereas LSCM demonstrates that molecules penetrate along shunt pathways deep into the skin tissues. The identity of these shunt pathways has not been established in the authors current work but most likely corresponds to hair follicles, sweat glands, or both.

On the Origin of Electroosmotic Forces in Human Skin

The rate of transport of acetaminophen, a neutral organic molecule, across skin is greatly influenced by the magnitude and sign of the iontophoretic current, a consequence of electroosmotic flow across skin. Figure 4 demonstrates that electroosmotic flow is operative locally within the shunt pathways in human skin identified in the previous section by SECM and LSCM. In these experiments, a SECM tip was placed in the receptor solution directly above a pore opening (after identifying the pore in an SECM imaging experiment) and used to monitor the concentration of acetaminophen as a function of the iontophoretic current, i_{app}. The values of concentration plotted in Fig. 4 were computed from the SECM tip current (Eq. 1) measured by oxidation of acetaminophen as it emerged from the pore opening.

Figure 4A shows a plot of the acetaminophen concentration, measured directly above a pore opening in full thickness human skin, as a function of i_{app}. A concentration of ~0.25 mM is obtained in the absence of iontophoretic current, corresponding to acetaminophen that is transported across the skin solely by diffusion. When i_{app} = +50 μA (positive current from the donor to receptor compartment) the concentration of acetaminophen at the pore opening increases by a factor of 4. Because acetaminophen is an electrically neutral species, the observed increase in concentration at the pore opening must result from electroosmotic transport. The increase in concentration for a positive valued i_{app} is consistent with electroosmotic flow through a negatively charged pore. When the current is reversed (i.e., i_{app} = −50 μA) the concentration of acetaminophen above the pore opening decreases to a negligible value, indicating that electroosmotic flow from the receptor solution to the donor solution (opposing diffusion) is sufficiently large to prevent acetaminophen from crossing the skin tissue.

The finding that electroosmotic flow occurs in shunt pathways in human skin is analogous to the previous finding that electroosmotic flow is localized in the hair follicles of hairless mouse skin (18). Thus, electroosmotic flow in shunt pathways seems to be a general phenomenon that occurs in human and animal skin. The authors specific interest here is to better understand the function of the shunt paths in generating the forces responsible for flow. A key question in

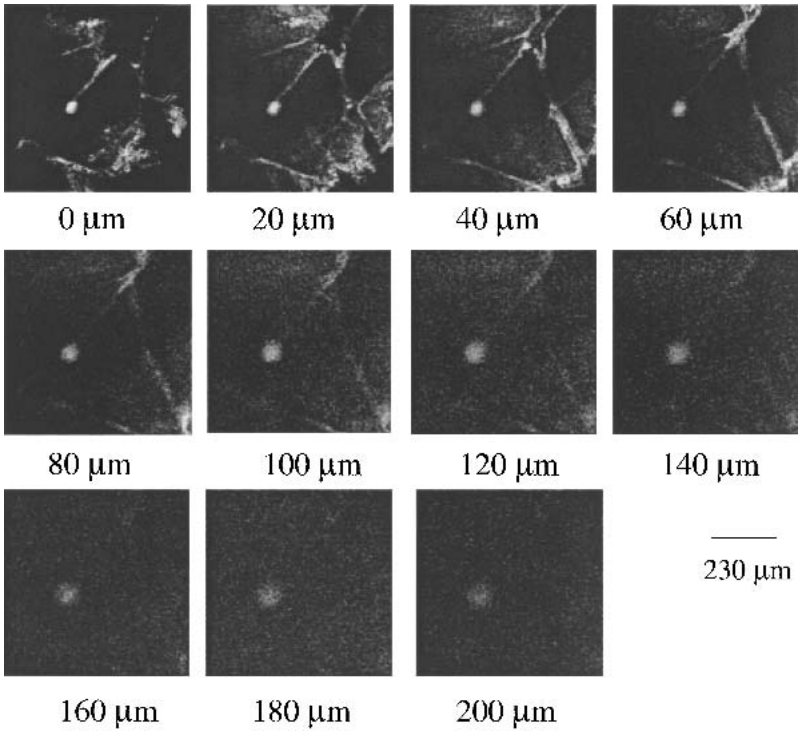

Fig. 3. Confocal microscopy images of full thickness human skin after iontophoretic transport of rhodamine 6G for 30 min at a current of 100 μA. The images illustrate the depth rhodamine 6G obtains during iontophoretic transport.

this regard is whether or not a microscopic pore opening in skin is sufficient for electroosmotic flow to arise or whether the flow originates deeper in the skin tissues. To investigate this issue, the authors repeated the above experiments using human stratum corneum that had been removed from whole human skin. Figure 5 shows an optical image of a region of excised stratum corneum in which a single pore opening is clearly evident. The sample of stratum corneum was mounted in the SECM/iontophoresis cell, and the concentration of acetaminophen at the pore opening was monitored as a function of i_{app} as described above in experiments using full thickness skin. As demonstrated in Fig. 4B, no measurable influence of i_{app} on acetaminophen concentration was observed using the stratum corneum sample, indicating that electroosmotic flow is inoperative in skin in the absence of the underlying tissues, specifically structures that form the shunt pathway through the dermal region. Thus, the concentration of acetaminophen measured in these experiments, independent of i_{app}, corresponds to acetaminophen that diffuses through the pore in the stratum corneum. The concentration of acetaminophen that results from diffusion across the stratum corneum is ~4 times larger than the corresponding value using full thickness skin. This result suggests that a significant diffusional barrier exists in the lower dermal region, consistent with diffusion in a pore before reaching the pore opening.

To better understand the abilities of full thickness skin and the stratum corneum to generate electroosmotic flow, a synthetic pore was fabricated by laser ablation in a thin layer of mica. The pore in the mica sheet is intended to mimic the pore opening in human skin. The resulting pore was ~17 μm in radius and ~100 μm long. Transport of acetaminophen through this pore was investigated before and after filling the pore with the cation-selective polymer, Nafion (26–28). Figure 6 shows the results of these experiments. When the pore is filled with the cation-selective material (Fig. 6A), the concentration of acetaminophen above the pore opening depends strongly on the applied iontophoretic current, a consequence of electroosmotic flow generated in the nanoporous and negatively charged Nafion phase. A 5-fold increase in the acetaminophen concentration is observed for $i_{app} = 50$ μA relative to the $i_{app} = 0$ μA value. Similar to results using excised stratum corneum, when the experiment is repeated for an empty pore (Fig. 6B), no electroosmotic transport is observed.

Overall, the synthetic mica/Nafion pore and the empty mica pore exhibits behaviors that are remarkable similar, respectively, to shunt pathways in full thickness human skin and the pore in human stratum corneum. The absence of electroosmotic flow in the empty mica pore and the stratum corneum pore can be rationalized in the small length-to-radius ratio of these pores. Ion selectivity and electroosmotic flow are both increased in pores that have small diameters (12). In addition, and by analogy with pressure driven laminar pore flow, fully developed electroosmotic flow likely requires pore lengths that are several times the pore diameter. The absence of flow, as evident in the results of Fig. 4B and 6B, suggests that these short pore structures do not support cation selectivity or electroosmotic flow.

Electroosmotic flow is clearly established at the time the mica pore is filled with Nafion. Cations migrate through the Nafion phase by interconnected and hydrated domains defined by negatively charged sulfonate sites (29). These nanometer scale porous regions in Nafion are known to give rise to electroosmotic flow (29). The complexity and uncer-

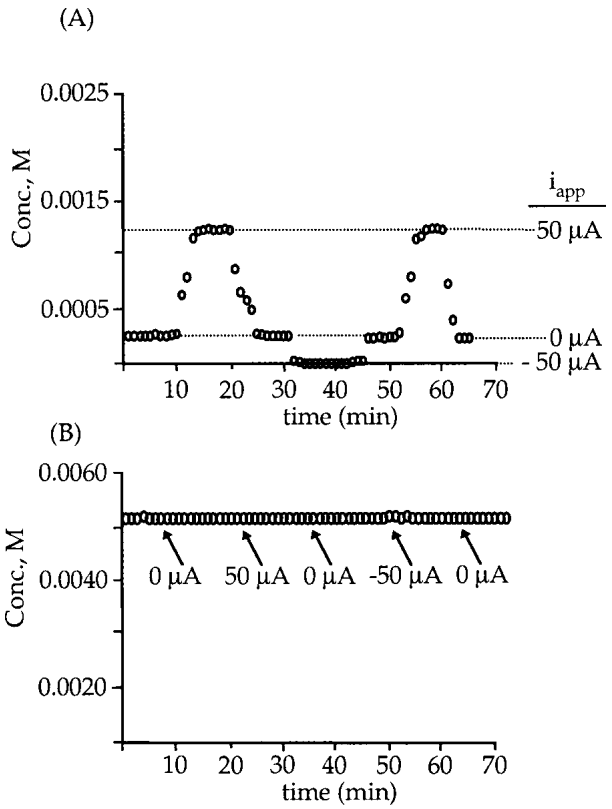

Fig. 4. (A) Plot of acetaminophen concentration directly above a pore opening in full thickness human skin as a function the applied iontophoretic current and time. The donor solution contained 0.2 mM acetaminophen; donor and receptor solutions contained 0.2 M NaCl. $i_{app} = 0$ corresponds to diffusive transport of acetaminophen across the skin. The concentration of acetaminophen is proportional to the local flux in the pore (see text). The increase in acetaminophen concentration when i_{app} is stepped from 0 μA to 50 μA is due to electroosmotic transport of acetaminophen from the donor to the receptor compartment. The decrease in acetaminophen concentration when i_{app} is stepped to –50 μA is due to electroosmotic flow from the receptor to the donor compartment, opposing the diffusion of acetaminophen. (B) Results of analogous measurements of a pore opening in human stratum corneum. The applied current, i_{app}, has no effect on the flux of acetaminophen, indicating that the pore opening by itself is insufficient to generate electroosmotic flow.

tainty of the structure of the shunt pathways in skin prevent a similar simple identification of the regions whereby electroosmotic flow is generated. It seems likely, however, that nano- or mesoscale porous phases that line the shunt pathway structures are responsible for the observed flow in full thickness human skin. The authors speculate that gap functions, which are abundant in Cx43 with carboxyl terminal groups, resemble meso-domains of negative charge that generate electroosmotic forces responsible for the observed flow along shunt pathways.

Quantification of the Pore Flux of Acetaminophen in Human Skin

A by-product of the SECM imaging experiments is the ability to quantify absolute values of molecular flux in the shunt pathways in skin and the synthetic mica membranes. As acetaminophen emerges from the shunt pathways during iontophoresis, it is transported into the receptor compartment solely by diffusion. Approximating the shunt pathway openings in skin and the mica membrane as disk-shaped sources from which the molecule is being emitted, the steady-state flux (N) away from these openings is obtained by solution of the mass continuity equation (24),

$$N = \frac{4DC(z=0)}{\pi a}$$

where a is the radius of the pore, $C(z = 0)$ is the concentration of acetaminophen at the follicle/receptor compartment interface, and D is the diffusivity of the molecule in the receptor solution. The diffusivity acetaminophen has been previously measured by electrochemical methods to be 9.1 ± 0.1 cm^2/s (18).

To evaluate N using Eq. (2), the radius of the pore, a, and the concentration of the molecule at the pore/receptor compartment interface, $C(z = 0)$, have to be measured. The latter quantities are obtained by determining the steady-state concentration profile, $C(z)$, in the receptor compartment directly above the hair follicle opening using SECM. For diffusion of molecules away from the follicle opening, the concentration profile along the x axis is described by Eq. (3) (24),

$$C(z) = \frac{2C(z=0)}{\pi}\tan^{-1}\left(\frac{a}{z}\right).$$

experimentally, this profile is established by measuring the SECM tip current $i_{lim}(z)$ at various heights above the center of pore opening, and converting $i_{lim}(z)$ to $C(z)$ using Eq. (1). Equation (3) is then fit to the $C(z)$ vs. z data to obtain best estimates of $C(z = 0)$ and a. The fitted parameters are then substituted into Eq. (2) to compute the flux. This procedure has been previously demonstrated to yield accurate values of individual pore radii and flux in synthetic membranes (17).

Steady-state values for C_s, a, and N corresponding to diffusion and iontophoresis from full thickness human cadaver skin and the Nafion-filled mica pore are presented in Table I. The values of N measured at 0 μA correspond to the diffusional flux, whereas diffusional and electroosmotic transport contribute to the flux at 50 μA. As the data indicate, the

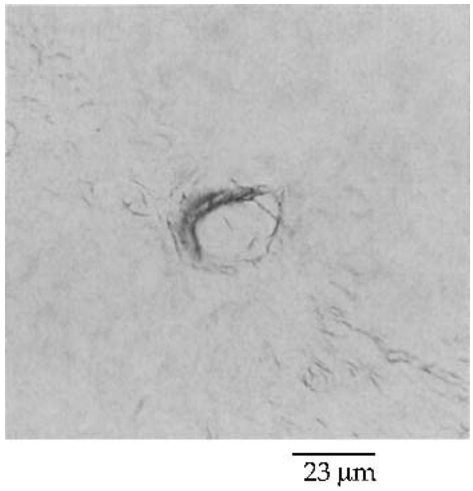

Fig. 5. Optical micrograph of a follicle or sweat pore opening in human stratum corneum. The stratum corneum was separated from human cadaver skin by soaking the skin in trypsin.

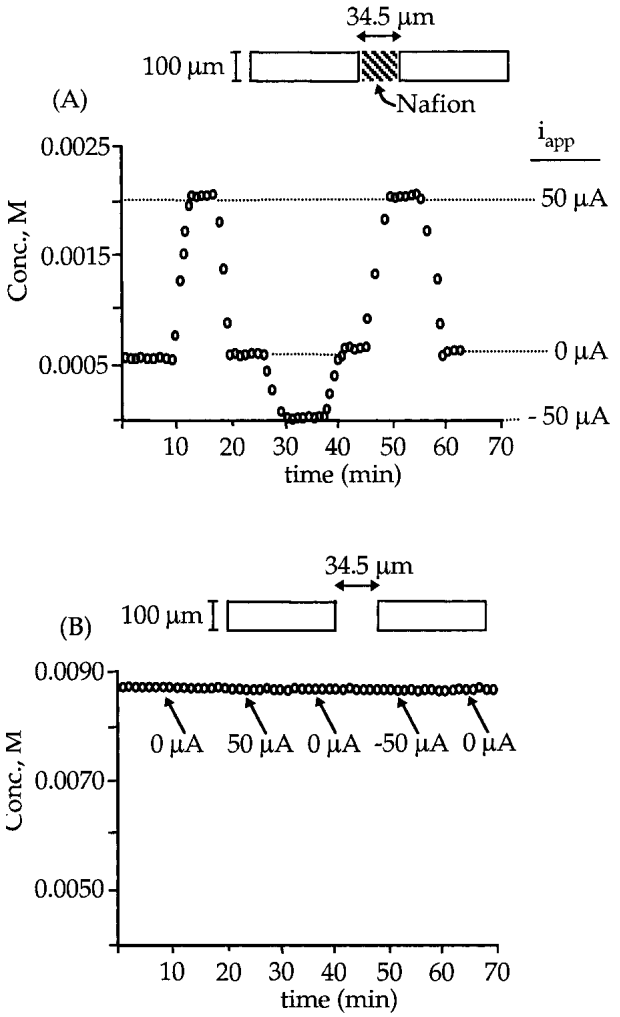

Fig. 6. (A) Plot of acetaminophen concentration above a Nafion polymer filled pore in mica (17-μm radius) as a function the iontophoretic current and time. Experimental conditions are the same as in Fig. 4. (B) Results of analogous measurements for a pore opening in mica in the absence of the Nafion polymer.

electroosmotic transport results in approximately a 5-fold increase in the transport of acetaminophen through the shunt pathways in human skin and the synthetic mica/Nafion pore. In a previous laboratory report, the flux of acetaminophen through hairless mouse skin was found to be 4.4 (± 1.0) × 10^{-10} mol/cm^{2}s for diffusion and 10.6 (± 1.1) × 10^{-10} mol/cm^{2}s at an iontophoretic current of 50 μA (18). These values are a factor of ~4 times smaller than the fluxes reported in Table I for human skin, suggesting that the rate of electroosmotic transport within shunt pathways in human and hairless mouse skin are comparable.

Table I. Diffusive and Iontophoretic Pore Flux

i_{app}	a (μm)[a]	C_s (10^{-6} mol/cm^3)[a]	N (10^{-9} mol/cm^{2}s)[b]
(a) Full thickness human skin			
0 μA	35.1	0.45	1.5
50 μA	35.1	2.1	7.0
(b) Mica/Nafion membrane			
0 μA	34.5	0.67	2.3
50 μA	34.5	3.2	11

[a] Determined by fitting eq. 3 to SECM measured concentration profiles above the pore openings.
[b] Computed from Eq. 2.

The use of steady-state approximation in the above analysis deserves comment. Because the measurements are made in a closed cell, there is a gradual change in the concentrations of acetaminophen in the donor and receptor compartments. The amount of acetaminophen transported during a measurement, however, is exceedingly small. For instance, the flux of acetaminophen through the 35.1 μm radius pore in full thickness human skin at 50 μA is 7 × 10^{-9} mol/cm^{2}s (Table I), resulting in the transport of 9.7 × 10^{-10} mol of acetaminophen over a 1 h period (a typical measurement time). The initial amount of acetaminophen in the donor compartment is ~7.5 × 10^{-4} mol (= 15 mL × 0.05 M). Thus, a negligibly change (~1 part per million) in the initial donor compartment concentration occurs during the SECM measurement, and the use of steady-state approximation is good.

CONCLUSION

The results demonstrate that electroosmotic transport of a neutral molecular species in human skin occurs within shunt pathways tentatively identified as hair follicles or sweat glands. The authors speculate that the presence of gap functions in epidermis surrounding these shunt pathways provides the porous charged domains necessary to induce electroosmotic flow. Iontophoretic transport through pore openings in the stratum corneum alone has been insufficient to induce electroosmotic flow.

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