Iontophoresis of a Model Peptide Across Human Skin *in Vitro*: Effects of Iontophoresis Protocol, pH, and Ionic Strength on Peptide Flux and Skin Impedance

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This study deals with effects of electrical (current density, frequency and duty cycle) and chemical (buffer pH and ionic strength) conditions on the flux of the octapeptide, 9-desglycinamide, 8-arginine-vasopressin (DGAVP), through dermatomed human skin. A pulsed constant current was applied during iontophoresis. The anode faced the anatomical surface of the skin samples inside the diffusion cells. The resistive and capacitative components of the equivalent electrical circuit of human skin could be calculated by fitting the voltage response to a bi-exponential equation. The skin resistance prior to iontophoresis varied between 20 and 60 $k\Omega.cm^2$. During iontophoresis a decrease of skin resistance and an increase of the series capacitances was observed, which were most pronounced during the first hour of iontophoresis; thereafter both quantities gradually levelled off to an apparent steady state value. The reduction of the resistance during iontophoresis increased non-linearly with increasing current density between 0.013–0.64 $mA.cm^{-2}$. The steady state resistance and capacitances did not vary significantly with frequency and duty cycle of the current pulse. There was no pH dependence of skin resistance at steady state. Between pH 4 and 10, the steady state peptide flux had a bell-shaped pH-dependence with a maximum of 0.17 $nmol.cm^{-2}.h^{-1}$ at pH 7.4, which is close to the I.E.P. of the peptide. Lowering the ionic strength from 0.15 to 0.015 M NaCl increased the steady state flux at pH 5 and pH 8 by a factor 5 to 0.28 ± 0.21 and 0.48 ± 0.37 $nmol.cm^{-2}.h^{-1}$, respectively. Together these observations suggested that DGAVP is transported predominately by volume flow. At pH 6, at which 65% of the peptide carried a net single positive charge, the steady state flux increased with increasing current density (0.013–0.64 $mA.cm^{-2}$) from 0.11 ± 0.03 to 0.19 ± 0.04 $nmol.cm^{-2}.h^{-1}$. Skin permeability during passive diffusion preceding iontophoresis at pH 6.0 was $2.9 \pm 0.6 * 10^{-7}$ $cm.h^{-7}$. In accordance with theoretical predictions based on the Nernst-Planck equation, to which a volume flow term was added, the flux was proportional to the mean voltage across the skin between 0.013 and 0.32 $mA.cm^{-2}.h^{-1}$. Variation of frequency or duty cycle did not result in significantly different peptide transport rates. From these studies it is concluded that DGAVP can be transported iontophoretically through human skin. The pH- and ionic strength-dependence of the iontophoretic peptide flux suggests that transport of DGAVP mainly occurs by volume flow. Furthermore, the flux of DGAVP appears to be controlled by the applied voltage rather than by the current density, as predicted by the Nernst-Planck equation.

KEY WORDS: iontophoresis; Nernst-Planck equation; percutaneous penetration; skin resistance; skin capacitance; peptide DGAVP; human skin.

INTRODUCTION

Percutaneous iontophoresis of peptide drugs is a promising alternative for oral or intravenous administration, mainly because metabolic degradation within the skin is often lower than in the gut and mucous epithelia. Also, iontophoresis produces large enhancement factors and the flux may be controlled by the electrical field applied across the skin (1). The flux resulting from iontophoresis may be caused by passive diffusion, electrical repulsion and electrically induced solvent flow (2,3,4). The concentration of the peptide and competing ions, the charge of peptide and skin, skin permeability to the peptide and the applied voltage are major factors, which determine the rate of transport (2). A theoretical approximation of the steady state flux is given by the Nernst-Planck equation, by assuming a uniform electrical field (Goldman-approximation) and including a term, which describes the volume flow contribution at steady state (5).

In the present *in vitro* study a pulsed constant current was applied and the voltage reponse was sampled, from which the component resistances and capacitances of the skin equivalent electrical circuit could be calculated. The flux of a model peptide, 9-desglycinamide, 8-arginine-vasopressin (DGAVP, Mw 1080, pK_{iso}:8.0) and the resistance-capacitance elements of human skin (pK_{iso}:3–4) were simultaneously measured with time as a function of pH, ionic strength of the buffer and of current density, frequency and duty cycle. The peptide is a fragment of arginine-vasopressin, has maintained the ability to improve memory-processes, but lost the potential to modify electrolyte metabolism and blood pressure (6). *In vitro*, it is a metabolically stable peptide with a half life of more than 12 hours in human plasma (7).

MATERIALS AND METHODS

Skin preparation Human abdomen skin was used within 24 hours after cosmetic surgery and dermatomized at a thickness of 300 μm. Skin slices of the appropriate thickness (300 μm) were selected after the thickness was determined by means of a micrometer and sandwiching the skin slices between two cover-glasses. The integrity was investigated by light microscopy and by measuring the resistance. Samples showing visible damage in the form of cracks and holes and/or having a resistance lower than 6 $k\Omega.cm^2$ were rejected.

Transport Cell The perspex diffusion cells contained a 1.5 ml donor and a 0.3 ml acceptor volume. The epidermal slices were mounted between the donor and receptor compartments, which were filled with buffer of the same composition. The skin was allowed to equilibrate to the buffer for two hours. Then the compartments were refilled with fresh medium, tubes and electrodes connected. The donor volume was stirred by circulating the donor solution at a rate of 15 ml per hour. A pump continuously supplied buffer from a reservoir via the acceptor chamber to a fraction collector, delivering 1-ml samples per hour. The entire set-up, including

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cells, pumps, tubes and reservoirs, was thermostated in an incubator at 32°C.

Electrodes The system was equipped with two separate sets of electrodes facing the apical and basal side of the skin: One pair of current injecting plate-electrodes and a second pair of bar-shaped voltage measuring electrodes. All electrodes were of the Ag/AgCl-type, and they were prepared by cleaning the silver-cores (99.9 and 99.999 % purity) with fine emery-cloth and coated electrolytically in a solution of 4 N HCl, with a layer of AgCl. The measuring electrodes were positioned at a distance of 1.5 mm from the skin surface. Ag/AgCl electrodes were selected to prevent electrolysis of water, thereby preventing pH changes. According to HPLC-chromatograms, no significant adsorption or electrolysis of DGAVP appeared to have been taken place at the anode or cathode surfaces, when during a period of 24 hours 0.1 mA direct current was injected by chlorided silver electrodes to a 1.4 μmol/ml solution of DGAVP in 0.15 M phosphate-buffered saline at pH 6.0 (2 ml total volume). NaCl depletion at the donor side during iontophoresis was limited to 10 % by connection of additional reservoirs to the donor circulating system.

Current delivery and voltage measuring device A computer controlled pulsed current source and a 20 MHz voltage sampling system were built at the Central Electronics Department of the Gorlaeus Laboratory (Leiden). The current source was programmed to provide a square-wave current of amplitudes ranging from 100 nA to 10 mA, frequencies between 0.25 Hz to 250 kHz and duty cycles (on/off ratio's) ranging from 255:1 to 1:255. The measuring electrodes were connected to the high input impedance (> 10 MΩ) of the voltage measuring system. The voltage was sampled with a frequency of 20 Mhz and 255 points per time base were collected, stored and analysed by the computer. The voltage sampling procedure required the selection of an appropriate time base, during which the descending part of the voltage could be accurately sampled. The computer used a least-squares fitting procedure to analyse the voltage decay.

The resistance of the electrode-electrolyte combination in the diffusion cells was measured, leaving the skin out. The voltage across electrolyte and electrode-electrolyte interface was fitted by a single-exponential equation. The resistance of the electrolyte was very low compared to skin. For example the resistance of the high ionic strength PBS was 51 ± 22 Ω, which was 100–1000 times lower than in presence of skin; the capacitance was 36 ± 9 nFarad, which is a 10–100 times lower value than in presence of skin. The resistance of the low ionic strength buffer was 343 ± 131 Ω and the capacitance was 3 ± 1 nF.

The voltage decay with skin present could be adequately fitted to a bi-exponential equation (equation 1), in which α and β are the relaxation times (RC-times). The corresponding skin equivalent electrical circuit could be modelled as a series connection of two parallel RC-circuits, a and b (8). The maximum voltages produced by current I across R_a and R_b are V_a and V_b. The capacitances C_a and C_b and the total skin resistance R_{tot} were calculated according to equations 3 and 4.

$$V(t) = V_a e^{-t/\alpha} + V_b e^{-t/\beta} + V\infty \quad (1)$$

$V\infty$ is a residual potential, defined as:

$$V\infty = \lim_{t\to\infty} v(t) \quad (2)$$

$$R_{tot} = R_a + R_b = \frac{V_a + V_b}{I} \quad (3)$$

$$C_a = \alpha/R_a \qquad C_b = \beta/R_b \quad (4)$$

Experimental protocol Each experiment consisted of two sequential parts: (a) An initial period of 12 hours during which the current was switched off, hence permitting only passive diffusion of the peptide, followed by (b) a final iontophoresis period of 6 hours during which the current was switched on. Every skin sample was oriented with its anatomical surface facing the anode, which was situated in the drug donor compartment. Skin resistance during the initial period was measured by applying a 10 μA alternating pulsed current of 100 Hz once every hour for 1.5 minutes. In the series of experiments, in which buffer pH and ionic strength was varied, a constant pulsed current of 100 Hz frequency, 50 % duty cycle and 0.20 mA amplitude was applied during the final period. A second series of experiments, in which either the mean current density, frequency or duty cycle was variable, was performed using a buffer of pH 6 and high ionic strength; mean current densities ranged between 0 and 0.64 $mA.cm^{-2}$, frequencies between 10 Hz and 10 kHz and duty cycles between 25 % and 99 %. Note that when the duty cycle was varied the amplitude of the current pulse was adjusted to obtain a constant mean current density of 0.13 $mA.cm^{-2}$. When varying the frequency the mean current density remained at a constant level of 0.13 $mA.cm^{-2}$.

Buffers Buffers of the same pH and ionic strength were used in the donor and acceptor compartment. The chemicals were reagent grade and solved in bidistilled water. Buffers were made with different pH's ranging from pH 3 to 10. The total buffer (citrate or phosphate) concentration never exceeded a concentration of 0.005 M. Citrate buffer was used in the range pH 3–5, while phosphate buffer was used in the range pH 6–10. The high ionic strength buffers contained furthermore 0.15 M NaCl. To make sure all the buffers were normalized to the same osmolarity, the low ionic strength buffers 0.015 M NaCl were supplemented with 0.285 M D(−)-Mannitol (BDH), giving an osmolarity of 305 mOsm. The pH of the donor compartment was checked after each experiment using pH-indicator paper (Merck). The pH between 3 and 8 appeared to be constant, but the high ionic strength phosphate-buffered saline, which had initially a pH of 10, had its pH changed during the course of iontophoresis at a mean current density of 0.13 $mA.cm^{-2}$ to 8, measured at the end of 6 hours of iontophoresis.

Peptide concentration and analysis 9-Desglycinamide, 8-arginine vasopressin dicitrate is an octapeptide of Mw 1412 (Mw is 1028 when dicitrate is omitted; the peptide was kindly supplied by Organon International, Oss, The Netherlands) and its I.E.P. is situated at pH 8.0. At pH 3 the peptide carries a net double positive charge, at pH 5 the charge is one positive and at pH 10 the peptide carries a net single negative charge. The peptide was dissolved in a concentration of 2.0 mg/ml (1.4 mM) in the donor buffer.

Peptide concentrations in the collected fractions were quantified by radioimmunoassay (9) or using a reversed-

phase HPLC-system. The system consisted of a Model SP8880 autosampler (Spectra-Physics Inc. San Jose, U.S.A.), a 4.6 × 25 cm Ultrasphere ODS column, with a particle size of 5 μm (Beckman Instruments, Mervue, Galwick, Ireland) and a Spectra 100 UV-Vis variable wavelength ultraviolet/visible absorbance detector (Spectra-Physics Inc., San Jose, U.S.A.) operating at 210 nm. The mobile phase was a 24:76 mixture of methanol/water and 0.01 M ammonium acetate (pH 3). The flow rate was 1.0 ml/min (pressure 1200 psi); and sample volumes of 100 μl were injected. Registration and data analysis were performed on SP4400 reporting integrator (Spectra-Physics Inc., San Jose, U.S.A.).

RESULTS

Skin resistance during passive diffusion During the passive diffusion period skin resistance was measured by sampling the voltage across the measuring electrodes for 1.5 minutes every hour using a 0.010 mA alternating pulsed current of 100 Hz, 50 % duty cycle. The resistance during the passive period had a constant value of 38 ± 16 kΩ (Fig. 1).

Skin resistances and capacitances during iontophoresis The resistance declined during prolonged current application. At the start of iontophoresis the decline was the fastest, and subsequently within 1–3 hours the total resistance levelled off to an apparent steady state value (Fig. 2A). Both the capacitances increased during iontophoresis. The increase was fastest at the start of iontophoresis and levelled off within 1–3 hours (Fig. 2B).

The steady state resistance dropped non-linearly from 38 ± 16 kΩ to 1.34 ± 0.52 kΩ by increasing the mean current density from 0 to 0.64 $mA.cm^{-2}$ (Fig. 3 A and B). The steady state values of C_a and C_b rose from 0 to 0.64 $mA.cm^{-2}$ (Fig. 3 A and B). The steady state values of C_a and C_b rose from (3.09 ± 3.08) * 10^{-8} and (3.20 ± 1.10) * 10^{-9} Farad at zero mean current density to (3.64 ± 1.21) * 10^{-7} and (1.10 ± 0.36) * 10^{-8} at 0.32 $mA.cm^{-2}$. Steady state resistance and capacitances neither varied significantly with frequency nor with duty cycle and pH; the steady state resistances were 9.8 ± 3.2 kΩ at 10 Hz and 6.0 ± 3.1 kΩ at 10 kHz and 9.9 ± 1.3 kΩ at 25 % duty cycle and 12.5 ± 7.1 at 99 % duty cycle.

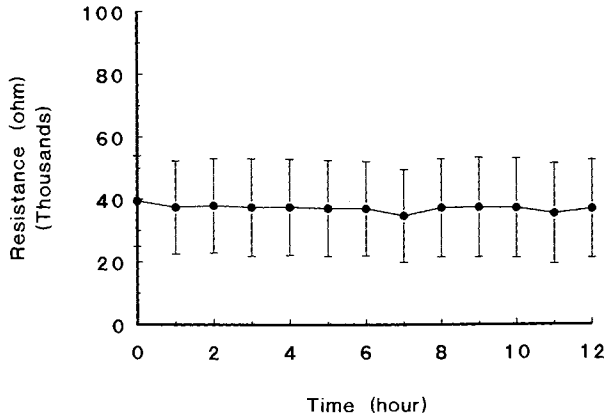

Figure 1. Resistance of dermatomed human abdominal skin during passive diffusion of DGAVP. The duration of passive diffusion was 12 hours. The buffer was PBS of pH 6.0. The thickness and area of the skin samples were 300 ± 50 μm and 0.785 cm^2 respectively. The resistance was determined once every hour, using an alternating current pulse of ± 10 μA amplitude and 100 Hz frequency.

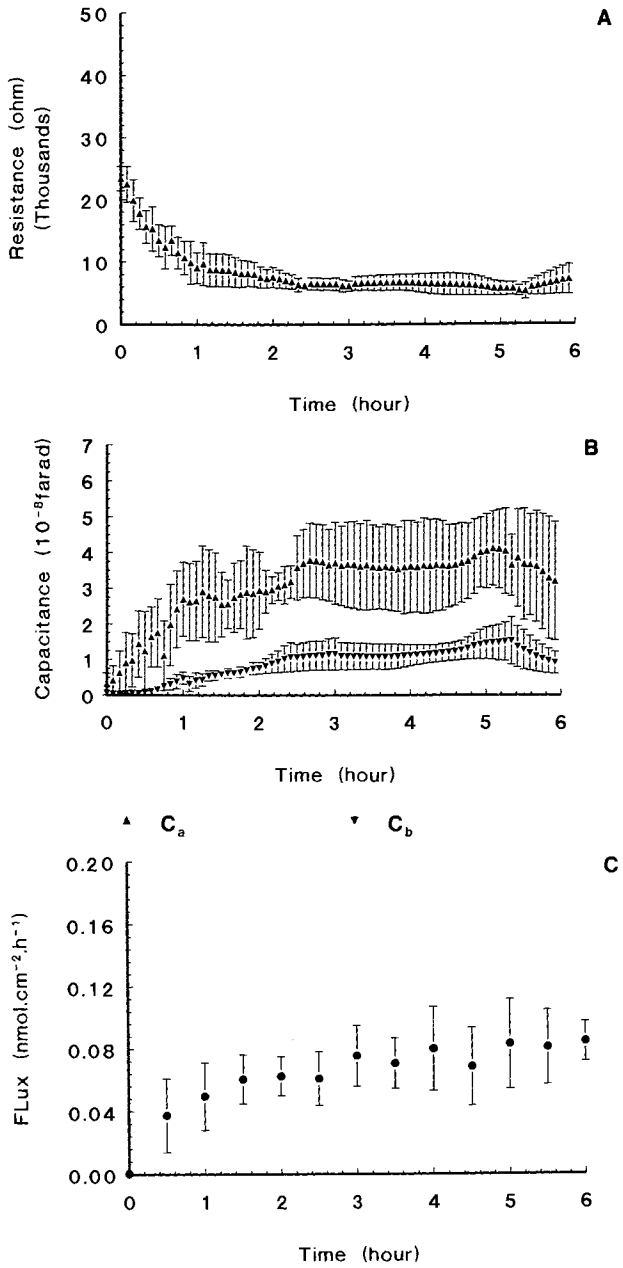

Figure 2. Resistance (A), capacitances (B) and DGAVP-flux as function of time during iontophoresis. Mean current density was 0.32 $mA.cm^{-2}$, frequency 100 Hz, duty cycle 50 %. The buffer was 0.15 M PBS of pH 6.0. The data represent mean ± STD of 6 experiments.

Peptide fluxes during passive diffusion and iontophoresis Prior to iontophoresis at pH 6 a passive flux of 0.40 ± 0.10 $pmol.cm^{-2}.h^{-1}$ was measured; the permeability of the skin to DGAVP was 2.89 ± 0.62 * 10^{-7} $cm.h^{-1}$. Steady state iontophoretic fluxes were obtained within 2–4 hours (Fig. 2c). The steady state flux has a maximum at pH 7.4 of 0.17 ± 0.1 $nmol.cm^{-2}.h^{-1}$ Fig. 4). Reducing the NaCl concentration at pH 5 and 8 from 0.15 to 0.015 M increases the flux by a factor 5 to 0.28 ± 0.21 at pH5 and 0.48 ± 0.37 $nmol.cm^{-2}.h^{-1}$ at pH 8 (Fig. 5). At pH 6, at which 65 % of

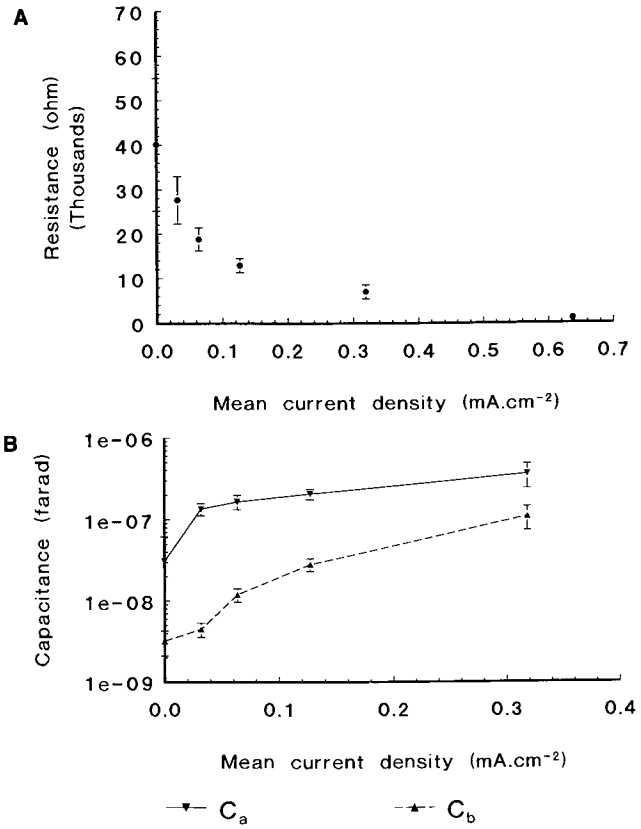

Figure 3. Skin total resistance (A) and capacitances (B) at steady state as a function of mean current density. The frequency was 100 Hz, duty cycle was 50 % and the buffer used was 0.15 M PBS of pH 6. Data represent mean ± S.D. of 6 experiments.

the peptide carries a net single positive charge, the steady state flux increased non-linearly with current density between 0–0.64 mA.cm^{-2}.h^{-1} (Fig. 6a). The flux was proportional (R^2 = 0.986) to the mean steady state voltage across the skin, except for the voltage resulting from the largest, 0.64 mA.cm^{-2}.h^{-1}, current (Fig. 6b). Neither the duty cycle

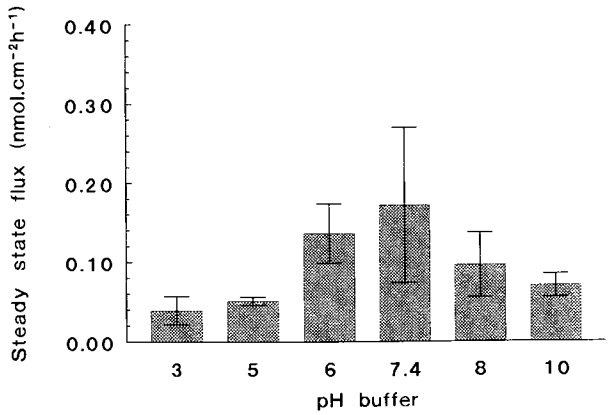

Figure 4. Steady state flux of DGAVP as a function of buffer pH. A 0.005 M citrate buffer was used at pH 3 and 5; a 0.005 M phosphate buffer was used in the range pH 6–10. The buffers contained also 0.15 M NaCl. The frequency was 100 Hz, the duty cycle was 50 % duty cycle and the mean current density was 0.13 mA.cm^{-2}. Data represent the mean ± STD of 5–6 experiments.

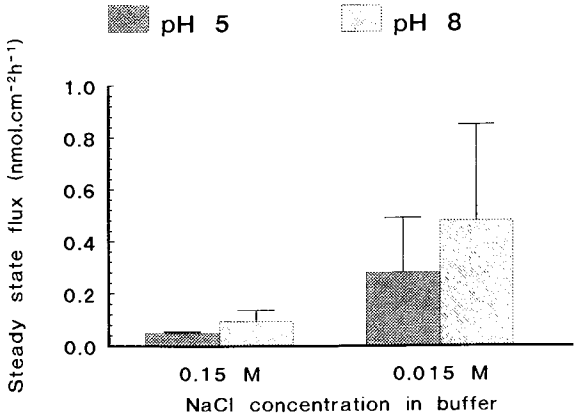

Figure 5. Steady state flux of DGAVP as a function of ionic strength. A 0.005 M citrate buffer was used at pH 5; a 0.005 M phosphate buffer was used at pH 8. The high ionic strength buffer contained 0.15 M NaCl; the low ionic strength buffer contained 0.015 M NaCl and 0.285 M mannitol. The frequency was 100 Hz, duty cycle was 50 % and mean current density was 0.13 mA.cm^{-2}. Data represent mean ± STD of 5–6 experiments.

nor the frequency significantly affected the steady state flux ($P \leq 0.05$): Steady state fluxes were 0.13 ± 0.04 nmol.cm^{-2}.h^{-1} at 25% duty cycle and 0.10 ± 0.03 nmol.cm^{-2}.h^{-1} at 99% duty cycle; they were 0.12 ± 0.02 nmol.cm^{-2}.h^{-1} at a frequency of 10 Hz and 0.12 ± 0.04 nmol.cm^{-2}.h^{-1} at 10 kHz.

DISCUSSION

In this study the effects of electro-chemical conditions on the flux of DGAVP and on human skin resistance and capacitances *in vitro* were investigated.

Effect of current density on skin resistance and DGAVP-flux Theoretical predictions of steady state fluxes through skin are mostly based on the Nernst-Planck equation. The equation can be solved, assuming a uniform electrical field (Goldman approximation) and a linear concentration gradient across the skin. Often a term Pe is inserted, which accounts for the flux resulting from the electrically induced solvent flow (5,11). The equation describing the flux of a positively charged species i then assumes the following form:

$$J_i = -P_i K [1 - (Pe/K)] \frac{c^a - c^d \exp(K [1 - (Pe/K)])}{1 - \exp(K [1 - (Pe/K)])} \quad (5)$$

in which K is a dimensionless driving force, given by $z_i F \Delta\phi / RT$, in which $\Delta\phi$ is the potential difference across the skin ($\Delta\phi^d - \Delta\phi^a$) and z_i is the valence. P_i is the skin permeability to species i, Pe is the Peclet number, Pe = v/P_i. v is the average solvent velocity, which is assumed to be proportional to the voltage gradient across skin (11). The concentration of compound i inside the skin at the side of the donor and acceptor compartment is c^d, respectively c^a. T is the absolute temperature, R and F are the gas constant and Faraday constant, respectively. At large positive potentials the exponential terms will dominate and the equation can be reduced to $J \approx P_i(K\text{-}Pe)$. At small potentials, the exponential exp[K-Pe] term can be written as 1 + [K-Pe] and the flux

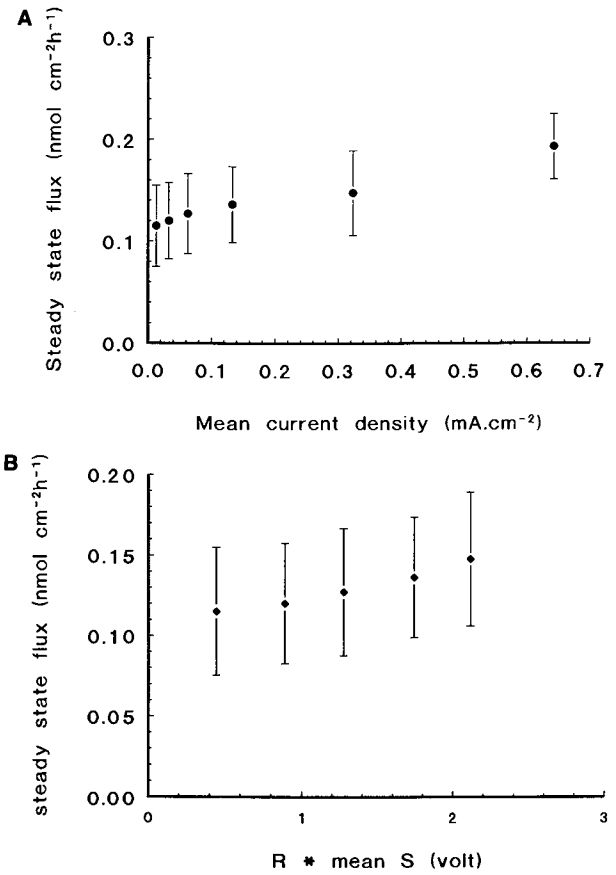

Figure 6. Steady state flux as function of mean current density (A) and voltage (B). The buffer used was 0.15 M phosphate buffered saline of pH 6.0. The current frequency and duty cycle were 100 Hz and 50 %, respectively. Data represent mean ± STD of 6 experiments.

equation reduces to $P[c^a\text{-}c^d\text{-}c^d(K\text{-}Pe)]$. Therefore, equation 5 predicts that the flux of a cationic species is proportional to the voltage gradient if the permeability P_i is constant. If a constant current is applied, the steady state flux will then depend, according to the theoretical predictions, on both current and skin resistance (Ohm's law). In our studies the resistance at steady state decreased non-linearly with increasing mean current density (Fig. 3A). The steady state flux between 0.013 and 0.32 $mA.cm^{-2}.h^{-1}$ still appeared to be proportional to the resulting voltage across the skin (R^2 = 0.986) (Fig. 6b), which is in agreement with predictions based on the Nernst-Planck equation.

Effects of buffer pH and ionic strength on DGAVP-flux Between pH 3–5, the net charge of DGAVP varies between 2 and 1 positive. If the major driving force in DGAVP iontophoresis would be electrorepulsion, the iontophoretic flux would be expected to have a maximum around pH 3–5. However in our studies the maximum was located at pH 7.4, which suggests that a large fraction of DGAVP is transported by means of volume flow.

The mean flux increased by reducing the concentration of sodium and chloride ions in the buffer at pH 5 and 8 by almost the same factor, around 4, which again suggests that the transport of DGAVP at pH 5 as well as pH 8 is dominated by volume flow. At pH 5, reduction of the concentration of these ions would have two effects: It would increase (a) the DGAVP transport number, which is the fraction of the current carried by an ionic species (10), and (b) the solvent flow (11). The latter is inversely related to the square root of the NaCl concentration, which changes by a factor 3.2 (12). At the isoelectrical point of DGAVP, pH 8, the enhancing effect can only be attributed to an increase of volume flow.

Conclusions The amounts of DGAVP delivered across human skin in vitro varies with current density, pH and ionic strength. The pH- and ionic strength-dependence of iontophoretic peptide flux suggests that transport of DGAVP mainly occurs by electrically induced solvent flow. Further, the flux of DGAVP appears to be controlled by the voltage across the skin rather than by the current density, as predicted by the Nernst-Planck equation.

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