Effect of Electroporation on Transdermal Iontophoretic Delivery of Luteinizing Hormone Releasing Hormone (LHRH) in Vitro

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Electroporation, the creation of transient, enhanced membrane permeability using short duration (microseconds to millisecond) electrical pulses, can be used to increase transdermal drug delivery. The effect of an (electroporative) electric pulse (1000 V, $\tau = 5$ msec) on the iontophoretic transport of LHRH through human skin was studied *in vitro*. Fluxes achieved with and without a pulse under different current densities (0- 4 mA/cm^2) were compared. The results indicated that the application of a single pulse prior to iontophoresis consistently yielded higher fluxes (5—10 times the corresponding iontophoretic flux). For example, at 0.5 mA/cm^2 fluxes were 0.27 ± 0.08 and 1.62 ± 0.05 μg/hr/cm^2 without and with the pulse, respectively. At each current density studied, the LHRH flux decreased after iontophoresis, approaching pre-treatment values. The results show that electroporation can significantly and reversibly increase the flux of LHRH through human skin. These results also indicate the therapeutic utility of using electroporation for enhanced transdermal transport.

KEY WORDS: transdermal drug delivery; LHRH; iontophoresis; electroporation; electropermeabilization; electrotransport.

INTRODUCTION

Drug delivery through the skin targeted to the systemic circulation—transdermal drug delivery (TDD)—has become a viable, attractive dosage form as evidenced by the success of some of the recent products (e.g., nicotine patches for smoking cessation, estradiol and other hormone replacement transdermal systems). Using iontophoresis (the movement of charged molecules through the skin due to a low-voltage electrical driving force), increased transport, compared to passive transdermal transport, of large molecular weight (> 800 daltons) compounds such as LHRH (1–4), TRH (5) and insulin (6–9), as well as small molecular weight compounds (lidocaine, etc.) (10,11) has been reported. However, iontophoretic delivery of therapeutic levels of large molecular weight compounds, such as peptides, still remains elusive primarily due to the impermeable nature of skin and the consequent inability to deliver therapeutically meaningful doses in humans.

Electroporation (also sometimes called electropermeabilization) has been used extensively in cell biology for many years to transport large molecular weight compounds (e.g., DNA) into the cell interiors (12). For DNA transfection into cells, a suspension containing the DNA and cells is placed in a cuvette containing two electrodes connected to a voltage source that provides a potential difference of about 1000 V (field≈1000 V/cm) across the electrodes. The condition typically results in a transmembrane potential (about 1 V) that lasts between μsec—msec. The mechanism underlying electroporation in artificial lipid bilayer membranes and liposomes has also been studied, and it has been hypothesized that the lipid bilayers are reversibly permeabilized by the formation of transient pores (13–17).

There have been many attempts to locate the permeability barrier in skin (18–24). It has been shown that a) pulsed electric fields reversibly permeabilize cell membranes which are predominantly bilayer lipids, and thereby facilitate the flux of large molecules, and b) multilamellar lipid domains of the SC are rate limiting for TDD. By combining these two observations, we have shown earlier that use of one electric pulse can increase skin permeability for large molecules (25).

Our objective for this paper was to examine whether electroporation increased skin permeability, by measuring drug flux under an iontophoretic driving force following the application of a single electrical pulse. Reversibly-increased membrane permeability is considered to be one of the hallmarks of electroporation. Hence, to assess reversibility, the passive flux was measured immediately before and for several hours after electroporation.

In this report, we compare LHRH transport under an iontophoretic driving force through electroporated and non-electroporated skin. We have used LHRH as an illustrative example for the following reasons: a) it is a large molecular weight (1182 daltons) compound, b) it is charged (+1 at pH 7.4) and c) there are literature reports which describe the iontophoretic delivery of LHRH (1,3,26,27), which enables us to compare our results with those of others. Furthermore, successful demonstration of the usefulness of electroporation in TDD of LHRH can then be extended to the delivery of other large molecular weight peptides.

MATERIALS

Skin

Full thickness human cadaver skin was obtained frozen from skin banks. Epidermis, which was the membrane used for all flux experiments, was heat-separated from full thickness skin. To separate the epidermis, a piece of full thickness skin with no obvious holes was soaked in water at 60°C for one minute and the epidermis teased off. The heat-separated epidermis was then stored frozen until the start of the experiment when it was checked visually for holes and obvious leaks. Epidermis from the same skin donor at contiguous anatomical sites was used in all experiments, thereby minimizing potential inter-donor variability.

Diffusion Cells

Side-by-side diffusion cells with two electrode ports in each half cell were used (LGA, Berkeley, CA). The donor and receiver compartments each had a volume of 3 ml and a

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transport area of 0.78 cm^2. The two outermost ports were 6 cm apart and the two inner ports were 3 cm apart.

Donor and Receiver Solutions

Isotonic phosphate buffered saline (PBS) was prepared by dissolving the salt (Dulbecco's PBS, GIBCO Laboratories, Life Tech, Inc., Grand Island, NY) in nanopure water. The pH was adjusted to 7.4 by adding NaOH or HCl. LHRH [pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2] (human, synthetic) was obtained as its acetate salt (Sigma Chemical Co., St Louis, MO) and was used as received. KCl (Sigma Chemical Co., St Louis, MO) solutions were made by dissolving the salt in nanopure water. ^3H-labeled LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was purchased from NEN (NEN Research Products, DE). Donor solutions were prepared by dissolving 2.5 mg of LHRH in 1 ml of PBS. This solution was spiked with a known amount of ^3H-labeled LHRH to yield specific activities of about 5 μCi/ml.

Electrodes

Silver wires were used as anodes. Ag/AgCl cathodes were made by electrochemically depositing a AgCl layer on a thin silver wire (0.1 mm dia, Aldrich Chemical Co., Milwaukee, WI). [For electroplating, a 5 cm long Ag wire was covered with a 3 cm heat shrink tube (Radioshack, CA) leaving one cm of Ag exposed at each end. This was then immersed in 0.5 N KCl and connected to the positive terminal of a constant current source. The counter electrode (cathode) was another silver wire. The electroplating current (0.25—4 mA amplitude depending on the current density of the experiment for which it was to be used) was passed for 12 hours]. Ag/AgCl electrodes were chosen for their electrochemical stability and their ability to maintain a constant pH in aqueous solution.

A constant current power supply (KEPCO, Flushing, NY) was used to provide the iontophoretic current. A multimeter (Fluke, Everette, WA) was used to measure the amount of current flowing through the skin.

A GENEPULSER™ (Bio-Rad, Richmond, CA) was used for generating the exponentially decaying electroporative pulse.

METHODS

All flux experiments were conducted at room temperature (≈20°C). Heat-separated human epidermis was sandwiched between two halves of the well-stirred four port diffusion cell. The donor solution (PBS containing labelled (^3H) and unlabelled LHRH) was added to the stratum corneum side of the diffusion cell (donor chamber) and PBS solution was added to the receiver chamber. The contents of the receiver chamber were removed periodically and the amount of radioactivity was quantified by liquid scintillation counting.

Silver wires, placed 6 cm apart, were used for pulsing (both anode and cathode); Ag anodes and Ag-AgCl cathodes were used for iontophoresis. LHRH has a net positive charge (+ 1) at pH 7.4. Therefore, to ensure electromigration through the skin, the anode was placed in the donor and the cathode in the receiver compartment of the diffusion cell. The experimental protocol involved measuring the passive flux for two hours. A single electroporative pulse was then applied, followed by 30 minutes of iontophoresis after which the LHRH concentration in the receiver was again measured. Finally, the passive flux was measured for another two hours to monitor recovery. Hence, the receiver fluid was emptied at 1, 2, 2.5, 3.5 and 4.5 hours after the start of the experiment; whenever the receiver compartment was emptied, it was refilled with fresh PBS. Electroporation was achieved using an exponentially decaying electric pulse with an initial amplitude of 1000 V and a time constant, τ, of 5 ± 1 msec. The time constant equals RC, where R is the load resistance (skin + solution) and C is the capacitance which could be set on the GENEPULSER. Skin resistance declines dramatically during the electroporative pulse and, hence, it is difficult to control τ, if the skin resistance (load) dictates τ. Therefore, during the pulsing, a 200 Ω resistor was connected in parallel to the diffusion cell. Hence, the net resistance was ≈ 200 Ω (since the load [skin + solution] was ≫ 200 Ω) and the capacitance on the GENEPULSER was set to 25 μF providing $\tau \approx 5$ msec. Following the electroporative pulse, the skin was exposed to 30 minutes of continuous DC at current densities from 0 to 4 mA/cm^2. A fresh piece of skin was used at each current density. For the corresponding iontophoretic experiments, an identical procedure was used except for the omission of the electroporative pulse.

Statistical analysis of the flux data was done using Student's t-test.

RESULTS

The iontophoretic transport of LHRH at 0.5 mA/cm^2 with and without an electrical pulse is shown in Figure 1. Prior to electrical treatment, the passive transport was about 0.05 μg/cm^2/hr for both pulsed and non-pulsed samples. The application of an iontophoretic current for 30 minutes resulted in a five-fold increase in LHRH flux (0.27 ± .08 μg/cm^2/hr; mean ± SD, n = 3) relative to passive transport. By contrast, the application of a single pulse prior to iontophoresis resulted in an average flux of 1.62 ± 0.05 μg/cm^2/hr (mean ± SD; n = 3). Following cessation of iontophoresis, the passive flux of LHRH decreased to 0.23 ± 0.1 (mean ± SD; n = 3) and 0.10 ± 0.3 (mean ± SD; n = 3) μg/cm^2/hr with

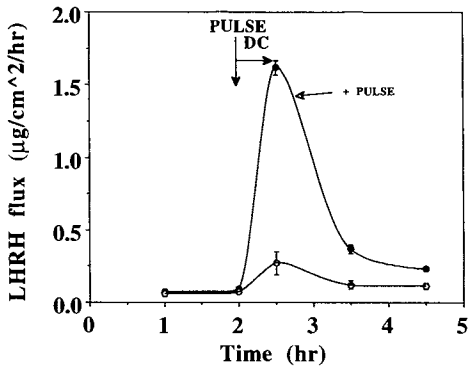

Figure 1: Iontophoretic transport of LHRH at 0.5 mA/cm^2 with and without an electrical pulse. The passive fluxes before and after the current treatment are also shown (0–2, 2.5—4.5 hrs). Key: ○—iontophoresis; ●—pulse + iontophoresis; mean ± SD.

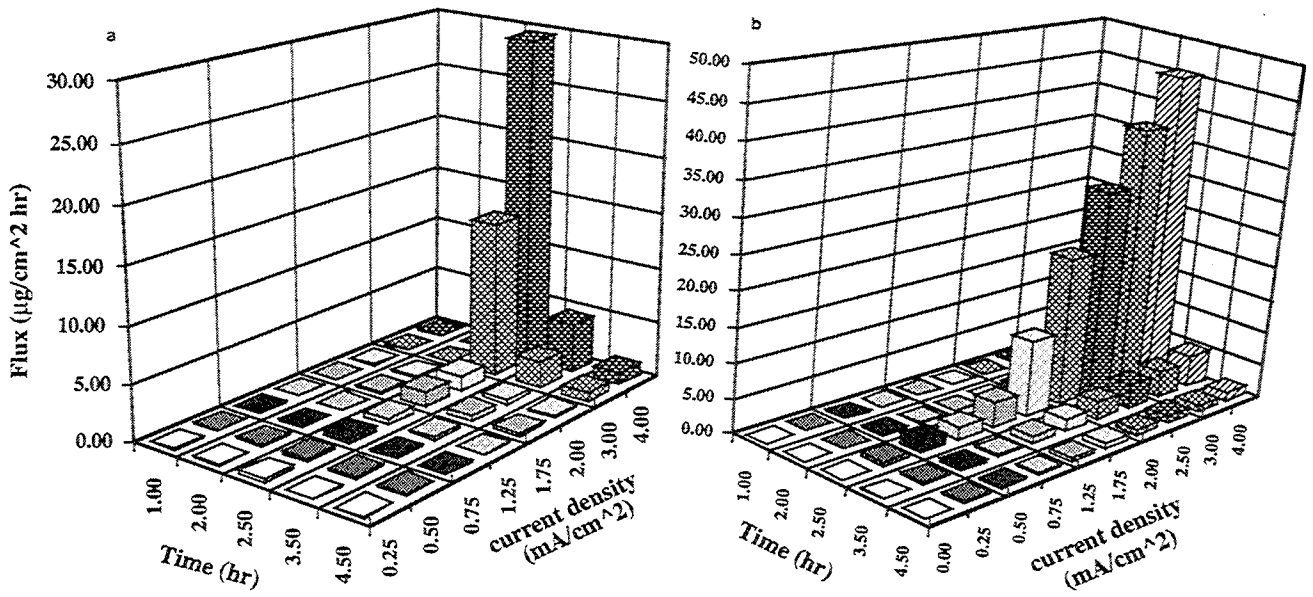

Figure 2: (a) The iontophoretic LHRH flux (mean) – before (1 -2 hrs), during (2—2.5 hrs) and after (2.5—4.5 hrs) iontophoretic treatment—shown as a function of current density. (b) Effect of an electrical pulse on the iontophoretic LHRH flux (mean) – before (1 -2 hrs), during (2—2.5 hrs) and after (2.5—4.5 hrs) electrical treatment—shown as a function of current density.

and without a pulse. Thus, the application of a single electroporative pulse resulted in the reversibly-enhanced iontophoretic transport of intact LHRH through human skin at a rate which was more than 30-fold greater than passive and five-fold greater than iontophoresis alone.

The experimental protocol was repeated at current densities ranging from 0 to 4 mA/cm^2. The results obtained without and with a pulse are shown in Figures 2a and 2b, respectively. In the absence of an iontophoretic current, the passive flux of LHRH was 0.02 ± 0.0 (mean ± SD; n = 3) μg/cm^2/hr prior to pulsing. During the 30 minutes immmediately after pulsing, however, the passive flux increased more than three-fold to an average value of 0.07 ± 0.01 μg/cm^2/hr (mean ± SD; n = 3), and then decreased to 0.04 ± 0.01 μg/cm^2/hr (mean ± SD; n = 3) during the following two hours. At all current densities studied, the application of a single electroporative pulse resulted in a statistically significant LHRH flux enhancement relative to iontophoresis alone. Finally, at each current density studied, the LHRH flux decreased after iontophoresis, approaching pre-treatment values. The flux obtained after pulsing, however, was consistently higher.

Examination of the results presented in Figures 2a and 2b show a current-dependent increase in LHRH flux during iontophoresis for both non-pulsed and pulsed samples. The iontophoretic flux versus current density data are plotted in Figure 3. These results show a non-linear increase in LHRH flux with increasing current for both pulsed and non-pulsed protocols. In all cases, however, the LHRH flux obtained after pulsing was significantly greater ($p < 0.01$) than that obtained in the absence of a pulse.

DISCUSSION

LHRH flux through human skin *in vitro* was measured before, during and after the application of direct current (0.5 mA/cm^2 for 30 min), either with or without (Figure 1) a single exponential electrical pulse (5 msec time constant, 1000V initial amplitude), applied at the initiation of the DC treatment. Prior to electrical treatment, the passive flux of LHRH was about 0.05 μg/cm^2/hr for both pulsed and non-pulsed samples. The application of DC (iontophoresis) for 30 min resulted in a significant increase in LHRH flux (0.27 μg/cm^2/hr) relative to passive delivery. The concentration of LHRH in the donor was 2.5 mg/ml, and, therefore, the "apparent permeability coefficient" (P = flux divided by donor concentration) was 1×10^{-4} cm/hr. A comparison with other experimental values (see Table I) shows that similar P

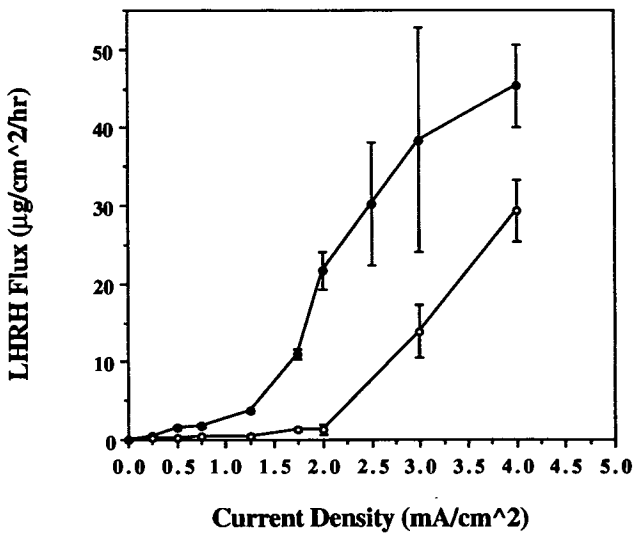

Figure 3: Iontophoretic flux of LHRH (mean ± SD) shows non-linear increase with current density with (●) and without (○) a pulse. $p \leq 0.01$ for all current densities (except 3.0 mA/cm^2, $p < 0.2$) when flux with and without pulse are compared.

Table I. Comparison of Values Reported in the Literature for the "Apparent Permeability Coefficient" for LHRH Iontophoretic Transport Through Skin

Reference	P (cm/hr)	Donor concn. (mg/ml)	Current density (mA/cm^2)	Skin type
Miller et al. (4)	4×10^{-3}	3.6	0.3	Hairless mouse skin
Heit et al. (1)	2×10^{-4}	1	0.2	Porcine skin flap
Srinivasan et al. (3)	2×10^{-3}	5 mg/ml	0.125–0.25 V	Ethanol pre-treated (2 hr) human skin
This work	1×10^{-4}	2.5	0.25–0.5	Human epidermis

values were obtained for the iontophoretic transport of LHRH through human skin and pig skin flap. These results demonstrate that while iontophoretic conditions were applied for only 30 minutes in our studies, the permeability coefficient obtained is comparable to steady-state values reported by others.

The same experimental protocol was repeated over a range of current densities from 0 to 4 mA/cm^2. The results obtained at each current density, either in the absence (Figure 2a) or presence (Figure 2b) of an electrical pulse, are qualitatively the same as the results obtained at 0.5 mA/cm^2 (Figure 1). An average passive flux (prior to electrical treatment) of 0.057 $\mu g/cm^2/hr$ (SD = 0.038; n = 76) was obtained for all samples evaluated. The passive flux of the LHRH analog leupron acetate was measured through human skin by several investigators. Lu et al. (26) found LHRH transport in human skin that was below the limits of HPLC detection. This value increased to 2.2×10^{-4} cm/hr when 10% urea was used as an enhancer. Srinivasan et al. (3) found that the passive transport was below their limits of detection, suggesting $P < 4 \times 10^{-6}$ cm/hr. When the skin was pretreated with ethanol for two hours, however, these investigators calculated $P = 6 \times 10^{-4}$ cm/hr. Thus, the passive transport of LHRH through human skin is very small, and the results presented here suggest an apparent permeability coefficient of about 10^{-5} cm/hr. The apparent permeability coefficient calculated from our experimental results is 10-fold less than values obtained with enhancers.

The application of a single, exponentially-decaying electrical pulse at the onset of iontophoretic treatment led to a significant enhancement of LHRH flux relative to that obtained in the absence of a pulse, at all currents studied (Figures 2a and 2b). The flux of any ionic species (J_i) with a charge Z_i is dictated by the applied current (i) and a proportionality constant called the transport number (t_i) by equation (1), where F is the Faraday's constant.

$$J_i = t_i \cdot i/Z_i \cdot F \quad (1)$$

Therefore, the amount transported is directly proportional to the charge transferred and governed by the same proportionality constant. The increase in LHRH transport due to an electrical pulse could, therefore, reflect the additional charge supplied by the pulse. Immediately after the pulse, the resistance of the skin drops from about 100 to about 1 $kOhm\text{-}cm^2$ (data not shown). Similar results have been reported by Prausnitz et al. (28). Assuming no potential drop across the solution or electrodes, the application of 1000V for 5 msec through a 1 kOhm resistor results in the transfer of about 5×10^{-3} Coulomb. By contrast, even at the lowest current density studied (0.25 mA/cm^2 for 30 minutes), the charge transferred through the skin is approximately 5×10^{-1} Coulomb. Hence, the contribution of the pulse charge to the enhanced transport of LHRH is, at most, 1%. Moreover, even in the absence of current, the application of an electrical pulse resulted in a significant increase in LHRH flux. It is likely, therefore, that the electrical pulse altered the intrinsic transport properties of human skin, regardless of the potential (electrical or chemical) driving the transport.

Following cessation of the electrical treatment, the passive flux was measured for both pulsed and non-pulsed samples. The results show that at all currents studied, the LHRH flux at two hours after iontophoresis decreased to a value significantly less than the maximal value obtained during iontophoresis. The value obtained for both protocols, however, was greater than the pre-treatment flux. Prausnitz et al. (28) have reported similar results; they measured calcein transport through human skin while continuously pulsing the sample. These investigators found that once the pulsing ceased, calcein flux decreased substantially over the next several hours. Thus, enhanced transdermal transport due to electrical pulsing is reversible on a time scale of hours. However, it should be noted that the measured post-treatment passive flux not only reflects transport through an altered membrane, but also the efflux of the LHRH which had accumulated in the skin during the current application (see below).

The ratio of the post-treatment flux to the pre-treatment value increased as a function of the applied current, regardless of pulsing. For example, for the experiments utilizing iontophoresis alone, the ratio increased from 1.7 at 0.25 mA/cm^2 to 50 at 4.0 mA/cm^2. The post-treatment flux (flux at 4.5 hrs) for the pulsed protocol was consistently greater (by an average of about three-fold) than the non-pulsed values at all currents tested. The difference, however, was only statistically significant ($p < 0.05$) at 0.25 and 0.5 mA/cm^2. These results demonstrate that for both protocols, the LHRH flux decreased following electrical treatment to a value which was strongly dependent upon the applied current.

The results in Figures 2a and 2b show that LHRH flux is strongly dependent on the applied current. The LHRH flux versus current density data obtained at low current densities ($< 1.25 mA/cm^2$) are plotted in Figure 4. These data show a linear dependence of flux upon current as described by equation 1. Linear regression analysis yielded transport numbers 4.3×10^{-5} and 4.5×10^{-6} in the presence or absence of a pulse, respectively. Thus, in this current range,

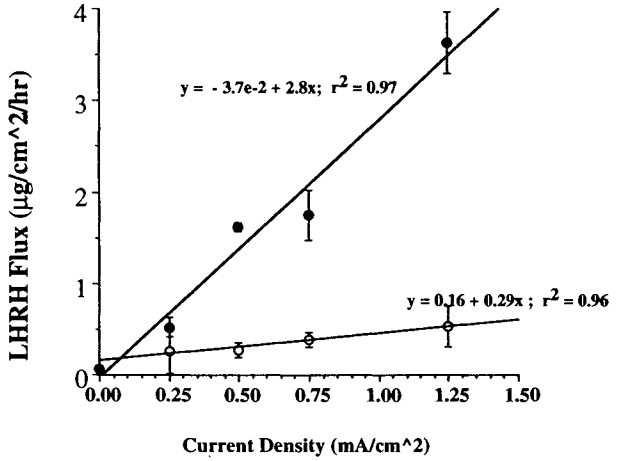

Figure 4: Data from Figure 3 replotted (mean ± SD) to highlight the linearity at low current densities (< 1.25 mA/cm^2).

the application of a single pulse prior to iontophoresis causes a 10-fold increase in the transport number of LHRH (t_{LHRH}). The transport number reflects the fraction of the total charge carried by the ion i, and it can be related to the ionic mobility of all species in solution through equation 2,

$$t_{LHRH} = C_{LHRH} \cdot U_{LHRH} \cdot Z_{LHRH}/\Sigma\, C_i \cdot U_i \cdot Z_i \quad (2)$$

where C, U, and Z represent the concentration, mobility and charge, respectively, of each ionic species. Since the charge and concentration of other ionic species are held constant in these experiments, the change in t_{LHRH} (due to the application of an electrical pulse) must reflect altered ionic mobility for LHRH relative to that of the other ionic species (primarily Na^+, Cl^- and other small ions). In other words, the application of a single pulse transiently altered the transport of properties of human skin. Furthermore, as discussed above, the passive permeability of LHRH through human skin is also enhanced by pulsing, again consistent with alteration of the intrinsic transport properties of the skin.

The post-treatment flux results presented here (Figures 1 and 2) and elsewhere (25) suggest that skin electroporation results in a high permeability state which lasts for hours, compared with much shorter times (seconds to minutes) in cell membranes. While this difference could be associated with longer-lived "electropores" in skin than those found in cell membranes, it is more likely that the results reflect LHRH binding to skin during iontophoresis. The subsequent desorption of peptide may then take many hours. Skin has a demonstrated reservoir capacity due to iontophoretic delivery (29), consistent with this hypothesis. The rate of flux relaxation is not substantially different between pulsed and non-pulsed experiments, again suggesting that pore resealing is not rate-limiting. Finally, a mechanism-based analysis of skin electroporation data suggests that the actual lipid resealing time is on the order of 10 minutes [Chizmadzhev et al.; in preparation].

The results presented in Figure 3 show a non-linear increase influx with increasing current, especially at high currents. While conventional transport theories based upon the Nernst-Planck equation predict that flux should be linear with current (equations 1 and 2) (30), the entire range of flux (J) and current (i) data from Figure 3, however, fits to an equation in the form $J = A\, i^2 + B$, where A and B are constants, with r^2 values for pulsed and non-pulsed experiments of 0.90 and 0.95, respectively. The energy delivered through a resistor (R) over time (t) is proportional to i^2. Therefore, the excellent fit of J versus i^2 suggests that flux is proportional to energy supplied. One possible mechanism by which electrical energy could influence iontophoretic transport is through Joule heating, similar to a mechanism proposed by other (31,32). Consistent with this model is the high post-treatment flux seen at high current densities. Regardless of the precise mechanism, however, the results presented here suggest that iontophoretic flux is proportional to the electrical energy delivered.

The results presented here show that electroporation can significantly and reversibly increase the flux of LHRH through human skin. The therapeutic utility of this enhanced transport is obvious from the data in Figure 1. The therapeutically acceptable limit of current for iontophoretic delivery is about 0.5mA/cm^2. The iontophoretic delivery of LHRH under the experimental conditions described here is less than 0.3 μg/cm^2/hr at this current. By contrast, an LHRH flux greater than 1.5 μg/cm^2/hr was achieved by the application of a single pulse to initiate iontophoretic delivery. An unacceptable current of greater than 2.0 mA/cm^2 would be required to deliver the equivalent mass of drug iontophoretically (see Figure 3). Hence, electroporation allows the enhanced delivery of peptides which cannot be effectively delivered by other transdermal means.

CONCLUSIONS

This study has shown that iontophoretic flux through electroporated skin is significantly enhanced compared to flux through non-electroporated skin. The fluxes achieved at current densities > 1.25 mA/cm^2 also suggest that membrane alterations induced by thermal changes can significantly enhance fluxes as well as the reversibility of the permeability changes. The effects of a range of electroporative conditions (different voltages and pulse widths) and the applicability of these *in vivo* need to be studied.

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