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Changes in the passive electrical properties of human stratum corneum due to electroporation

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Abstract

The stratum corneum (SC) is the main barrier to molecular and ionic transport across mammalian skin and has been extensively studied by others at low voltages ($U_{skin}(t) < 10$ V) in order to partially characterize the skin. Here we used one or more exponential pulses ($\tau_{pulse} = 1$ ms) and a temperature of 25 ± 2°C and found that the low voltage passive electrical properties (impedance) change rapidly and significantly if these pulses result in $U_{skin,0} > 40$ V. In contrast, the dynamic resistance (describing passive electrical behavior in a nonlinear range) changes dramatically by application of pulses between 40 V and 80 V and then it settles at levels between 50 Ω and 100 Ω. We also found that recovery of the low voltage electrical parameters after pulsing depends mainly on the voltage, and, for multiple pulse protocols, on the number of pulses. For single pulses of $U_{skin,0} \approx 90$ V or less the electrical recovery was almost complete, returning to within 0.90 of the pre-pulse value. In contrast, larger pulses result progressively in decreased recovery. The recovery for pulses > 90 V revealed several characteristic times, suggesting the involvement of different processes. For multiple pulses with $U_{skin,0} > 130$ V almost no recovery of the transdermal resistance, R_{skin}, was evident (returning to < 0.10 of pre-pulse values), i.e., essentially permanent changes in the stratum corneum occurred. This is similar to that of single bilayer membrane electroporation, for which a transition from reversible to irreversible behavior occurs as transmembrane voltage is increased. Thus, these results are consistent with the hypothesis that 'high-voltage' pulses cause electroporation within the SC, i.e., that elevated transmembrane voltages result in creation of new aqueous pathways ('pores') across SC lipid regions.

Keywords: Electroporation; Iontophoresis; Stratum corneum; Passive electrical properties; Impedance; Dynamic resistance; (Human skin)

1. Introduction

The main barrier of mammalian skin to the transport of ions and molecules, particularly charged molecules, is its outermost layer, the stratum corneum (SC) [1,2]. The SC is a heterogenous, dead layer about 10 to 15 μm thick and consists of flattened remnants of cells (corneocytes) and about one hundred lipid bilayer membranes arranged in series [3,4]. There are two general motivations for investigating the use of 'high-voltage' pulses to increase molecular transport across the skin [5]: (1) transdermal drug delivery, which is presently limited to small, lipophilic compounds [6], and (2) non-invasive sampling for diagnostics in which analytes are to be extracted for external assay. It has been proposed that a large electric field across the SC lipids leads to creation of aqueous pathways [7,8] and simultaneously provides a local driving force for transport through these pathways, i.e., electroporation of the SC occurs.

Recent studies have shown that the transport of charged molecules ranging in molecular weight from about 400 g/mol to 1600 g/mol was tremendously enhanced by high voltage pulsing [7,8]. Ordinarily such molecules penetrate the skin negligibly. In addition, accompanying mea-

Abbreviations: A_{skin}, area of the skin exposed to the electrical protocol; $I(t)$, current through the entire system as a time function; $f(t)$, time function; R_{bulk}, resistance of the saline and electrodes in series; R_{dy}, dynamic resistance (Ω); R_{in}, membrane resistance; R_{meas}, measurement resistor (10 Ω); R_{sal}, resistance of the saline between the inner electrodes; R_{skin}, dc-part of the skin's impedance; r, pore radius; r_{eff}, effective radius of a calcein molecule; r_{min}, minimum pore radius; SC, stratum corneum; $U_{inner}(t)$, voltage at the inner electrodes as a time function; $U_{outer,0}$, maximum voltage, applied at the outer electrodes; $U_{skin,0}$, amplitude of $U_{skin}(t)$; $U_{skin}(t)$, voltage across the skin as a time function; a,b,c, constants, used in Eq. (3).; τ_1, τ_2, time constants, used in Eq. (3).; C_a, C_b, R_a, R_b, R_{skin}, circuit elements for the skin model; ϵ_l, relative permittivity of lipids; ϵ_w, relative permittivity of water; τ_{pulse}, time constant of the exponential decaying pulse.

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surements of the transdermal resistance, R_{skin}, showed that a large rapid decrease occurred. Both changes are consistent with electroporation.

Electrical measurements are particularly attractive for quickly assessing both the degree of aqueous pathway creation, and the extent of barrier recovery. In both cases the electrical measurements primarily reflect the transport of small ions, usually Na^+ and Cl^-. This transport presumably involves aqueous pathways, because the electrostatic energy change associated with inserting even a small ion into a lipid region is large [9]. The passive electrical properties are relatively straightforward to measure and are sensitive to several property changes in biological material. In general, passive electrical properties at $U_{skin}(t) < 10$ V appear to involve unchanged structure of the skin, a view which is strongly supported by the recent work of Dinh et al. [10].

The electrical behavior associated with conventional iontophoresis conditions ($U_{skin}(t) < 10$ V) is of great interest, because of the need to understand the basic mechanisms of molecular transport for transdermal drug delivery by iontophoresis [10–17]. The experiments and theoretical model of Dinh et al. are particularly relevant, as they show that both conductive (electrical drift) and convective (field induced flow) through fixed aqueous pathways ('pores') contribute to ionic transport. They also show that the relatively slow (seconds to one hour) resistance change is due to a change from predominantly conductive transport at the smaller voltages to predominantly convective transport at the larger iontophoretic voltages. These impressive results provide an explanation of the significant but slow changes in resistance that occur, without any need to invoke structural changes within the SC. They are consistent with a simple, fixed cylindrical pore with radius of about $r = 2.5$ nm, which is close to the values found by others. Thus, the overall conclusion is that small ion transport through fixed aqueous pathways can change from predominantly conductive transport at the lowest voltages to predominantly convective transport at the highest iontophoretic voltages, and that this gradual transition without SC structural changes accounts fully for the main electrical changes for $U_{skin}(t) < 10$ V.

In marked contrast, electroporation is known to dramatically change the electrical resistance of lipid-based barriers, viz. artificial bilayer membranes [18,19] and cell membranes [20,21]. More recently electroporation has been suggested as being responsible for the rapid and large electrical changes that occur because of 'high-voltage' pulsing of tissues [8,22]. Under similar conditions the time-dependent transmembrane voltage across a single bilayer membrane can be understood in terms of conduction of small ions through electrically created aqueous pathways ('pores') that perforate a bilayer membrane [19,23]. For the single bilayers used in conventional artificial planar bilayer membrane experiments and in cell experiments, the electrical behavior is consistent with a transient aqueous pore model in which (1) an elevated transmembrane voltage results in a non-linear increase in the production and expansion of pores, and (2) the voltage is limited to about 1.5 V because of the strong, non-linear increase in bilayer membrane conductance associated with hindered motion of small ions. In contrast, little was known about the dynamic behavior of the electrical conductance at 'high voltages' ($U_{skin,0} > 50$ V) for a complex multilamellar bilayer system such as the SC. In addition to several recent experiments a theoretical model for electroporation of the stratum corneum has been proposed (Chizmadzhev, unpublished data).

2. Materials and methods

2.1. General

The overall aim of this study was to investigate more fully the large and very rapid electrical changes that are caused by 'high-voltage' pulsing, with an emphasis on using pulses that are the same or similar to those used to cause large increases in molecular transport across skin preparations. We therefore focused on the behavior of heat stripped SC, which was obtained from human cadaver skin by established methods [24]. After preparation the skin was stored on waxed paper in a refrigerator at high humidity in order to keep in proper condition for up to several days. A typical thickness of these preparations was about 50 μm, i.e., 10 to 15 μm for the stratum corneum and about 35–40 μm for the epidermis. The area of the skin exposed to the electric field (A_{skin}) was 0.71 cm^2.

During the experiment the skin was maintained at room temperature (25 ± 2°C) while clamped in a side-by-side flow-through permeation chamber (Fig. 1a). In related molecular transport experiments (not described here), a fluorescent molecule was provided in the donor compartment of the chamber, and contacted the SC, while the internal epidermis surface faced the receptor side. During the prepulsing phase a skin specimen was clamped in the permeation chamber, and the dc-resistance (R_{skin}) was measured. Initially R_{skin} changed significantly because of hydration but reached a steady value (50–150 kΩ), usually within about 10–30 min. No pulses were applied until R_{skin} was essentially constant. Moreover, a particular skin preparation was not used if the resistance was too low ($R_{skin} < 20$ kΩ).

2.2. Voltage across the skin U_{skin}

The voltage across the inner electrodes, U_{inner}, and the total current through the entire system, $I(t)$, were both measured, and the time-dependent transdermal voltage, $U_{skin}(t) = U_{skin,0} \cdot f(t)$, was then calculated using

$$U_{skin}(t) = U_{inner}(t) - I(t) R_{sal} \quad (1)$$

where R_{sal} is the resistance of the saline between the inner electrodes. Our equipment for measuring the voltage during the pulse had a bandwidth of 200 kHz, and therefore the first 10 μs could not be measured reliably. For this reason, for calculating $U_{skin,0}$, we used the time interval between 100 μs and 500 μs to extrapolate the voltage back to the beginning of the pulse assuming an exponential decay of the pulses. Because of this limitation it was impossible for the first 10 μs to distinguish between SC-dependent events (e.g., pore formation) and the behavior of the high voltage probe used for the measurement.

2.3. Resistive behavior during the application of high voltage

Electrical impedance is defined only for electrical elements with a linear, time-invariant response [25], but the

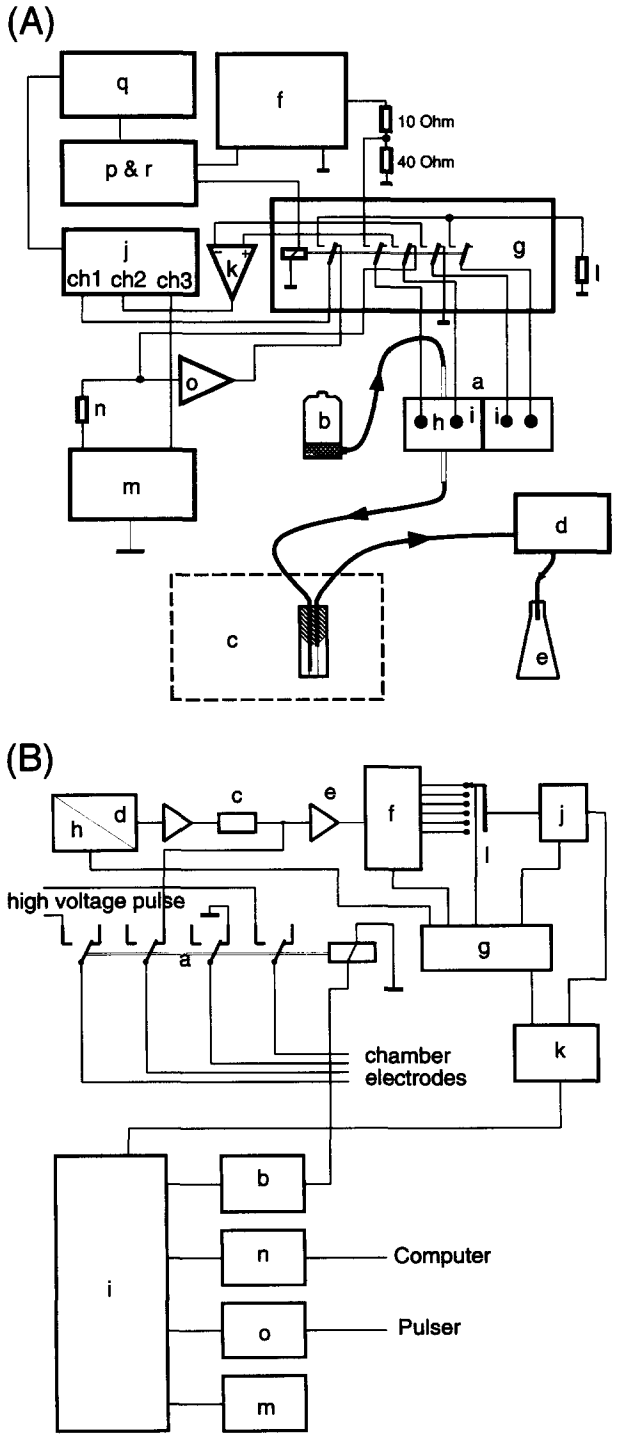

Fig. 1. (A) Apparatus for simultaneous measurement of molecular transport and passive electrical properties of a tissue, here preparations of human skin. The main component of this system is a side-by-side flow through chamber (a), connected in a continuous sampling system (supply dish (b), spectrometer (Fluorolog-2, model F112Ai, SPEX Industries, Methuen, NJ) (c), peristaltic pump (d) and waste dish (e)) in order to determine the transdermal transport of fluorescent molecules (i.e., calcein) due to 'high voltage' pulsing. The chamber holds four electrodes (Ag/AgCl, in vivo metrics, Healdsborgh, CA), two each at the receptor and the donor side. The 'high-voltage' pulses were delivered either by a GenePulser (Bio-Rad, Richmond, CA) or a ECM 600 (BTX, San Diego, CA) (f) and via high voltage switch unit (g, indicated here for impedance measurement between pulsing) applied at the outer pair of the electrodes, with the positive electrode in the receptor side (h). In order to control the time constant of the exponential pulse a voltage divider 10 Ω/40 Ω was placed at the output of the pulser. During the pulse the resulting voltage at the inner electrodes (i) (distance from the skin about 11 mm) was recorded by an oscilloscope (HP 54602 A, Hewlett Packard) (j) at channel 2. A special differential amplifier was used to match chamber, switch and oscilloscope together. The voltage drop at the serial resistor (l) was recorded with channel 1 in order to calculate the total current through the chamber. Between the pulses the inner pair of electrodes was used to measure the passive electrical properties. A function generator (m) (HP 3411, Hewlett Packard) delivered a continuous rectangular wave with negligible bias, a peak to peak voltage of 150 mV and a frequency of 1.25 kHz. The output of the generator was matched to 10 kΩ, using an amplifier and a measuring resistance R_{meas} (n) of 10 kΩ. The voltage gain was 1 and the frequency commenced in the range of 100 Hz to 200 kHz (−3 dB). This voltage was applied to the chamber electrodes. The resulting deformed wave across the chamber was amplified (o) and recorded on channel 1 of the oscilloscope. The trigger for this signal was provided by the sync.-output of the generator at channel 3. The entire experiment was controlled by a microcontroller board (8052 at MiniCon52, Phytec, Mainz, Germany) (p), and the data for later analysis, using a SPARC 10-station (SUN-Microsystems) were stored in a PC (q) (Bell Technologies, AT-model). For rapid electrical measurements a custom made apparatus (r) was used together with the microcontroller board (p). (B) Apparatus for rapid electrical measurements. Between the pulses the high voltage relay (a) (relay interface (b)) contacts the inner electrodes in series to the measuring resistance (c) and the generator (d). The generator delivers 1 or 3 kHz rectangular waves, where usually 3 kHz was used, with a voltage of 112 mV with no bias. The resulting wave form is amplified (e) and sampled at 6 times (in 3 kHz regime (2.62 μs, 10.84 μs, 52.32 μs, 102.5 μs, 152.2 μs, 318.9 μs after the rising edge), using 6 identical track and hold amplifiers (f). The time base controller (g) for the sampling times is maintained by the mother generator, oscillating at 12 MHz (h). The output of the track and hold amplifiers is entered by the microcontroller (i) (8052) via analog digital converter (j) (ADC) and a PIO (8255, parallel in-output) (k), which also switches (l) the input of the ADC. The data were stored in nonvolatile RAM (m) in order to transfer it after the experiment, when the time is not critical via RS-232 interface (n) to a PC for further analysis. The apparatus also provides an interface to control the pulser (o), allowing control of the trigger of the pulser and reading an EOP (end of pulse signal). In the case of the Bio-Rad GenePulser an additional feature is that the voltage of the pulses can be controlled *via* this interface. For this reason both pulsers, Bio-Rad and BTX, were modified internally.

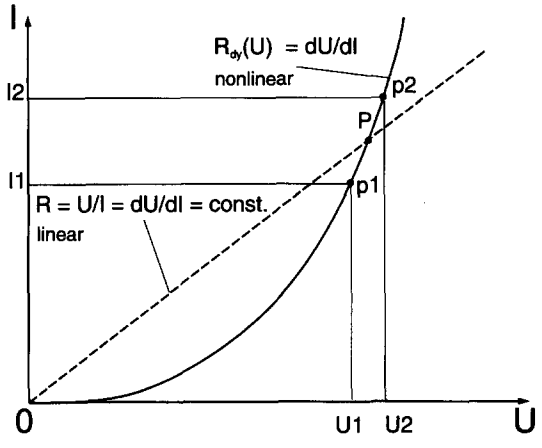

Fig. 2. Illustration of the $U-I$ characteristic of a nonlinear system. The resistance is the slope at each point of the curve. Simply by calculating U/I at the operating point P the results are misleading (dashed line). The dashed line indicates the simple quotient U/I in the operating point, while the solid line is the real $U-I$ characteristic. As seen in the figure the difference can be important. Thus, the dynamic resistance is used in order to describe the passive electrical properties of a nonlinear system as a function of U. Skin exhibits a linearity below voltages of about 300 mV, and thus in this range the R_{dy}, supposing time invariance, is nearly equal (if the change in $U_{skin}(t)$ and $I(t)$ is slow, compared to the charging time of the skin) to the dc-part of the electrical impedance, here referred to as R_{skin}.

skin resistance is nonlinear for $U_{skin}(t) > 300$ mV, i.e., Ohm's law breaks down [26]. In such cases a simple calculation of resistance yields complicated misleading results (Fig. 2). Moreover, the rapid creation of new aqueous pathways, probably on a time scale which is small compared even to the charging time of the skin (20 to 100 μs), leads to the expectation of a strongly time dependent system. With this in mind, a dynamic resistance, R_{dy}, was introduced, where R_{dy} is a function of both the time and the applied voltage:

$$R_{dy} = dU_{skin}(t)/dI(t) \quad (2)$$

i.e., the slope of the U–I-relationship (Fig. 2). It has the dimension of resistance, Ω. In the special case of linearity of the skin (at $U_{skin}(t) < 300$ mV) and slow changes of $U_{skin}(t)$, R_{dy} is equal to the ordinary dc-part of the impedance, R_{skin}.

2.4. Impedance between pulsing

The skin's low voltage electrical properties were measured between pulses using the inner pair of electrodes (Fig. 1a). The use of phosphate-buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO) provided a constant concentration of Cl^- and resulted in negligible electrode polarization effects for the frequency range 1 kHz to 100 kHz. In order to provide good time resolution, a time domain measurement was used (Fig. 1). The series combination of the skin and the saline and electrodes was connected in series to a 10 kΩ non inductive measurement resistance, R_{meas}. This voltage divider was excited by a symmetric rectangular wave with a frequency of 1.25 kHz, a peak to peak voltage of 150 mV and zero bias. The rise and fall time was less than 50 ns.

The voltage trace across the skin specimen was deformed because the skin's impedance was not entirely ohmic. It was recorded by the digital oscilloscope. Approx. 350 ms were needed to acquire data from the oscilloscope, which resulted in a limited time resolution of the system, and for this reason only three measurements per second were possible. For skin not subjected to 'high-voltage' pulses, the deformed pulse fits well a sum of two exponential functions

$$U_{skin}(t) = ae^{-t/\tau_1} + be^{-t/\tau_2} + c \quad (3)$$

with time constants $\tau_1 \approx 100$–800 μs and $\tau_2 \approx 20$–60 μs. These time constants also depend on the measuring resistance, R_{meas}, and therefore are not the intrinsic time constants of the SC. In order to interpret the data, a circuit model with five elements (three resistances and two capacitances; Fig. 3) was chosen. This particular model was chosen for two reasons: (1) a plausible association of each circuit element could be made with a part of the measured system, and (2) each element can be determined reasonably well from the deformed pulse. Calculations which yielded the values the circuit elements proceed in two steps: (1) numerically fitting both exponentials, which yielded the associated time constants, and (2) evaluating the circuit elements. The overall procedure took the following into consideration: the bulk resistance of the saline, an average voltage drop across the electrodes, the excitation voltage, and the resistance R_{meas}. As shown below, this model allows quantitative description of the recovery after electrical pulsing.

2.5. Impedance measurement with higher time resolution

In order to address the problem of inadequate measurement capability over short times, another apparatus (Fig. 1b) was used to make faster measurements immediately after pulsing. The principle is the same as described above, but this system uses a square wave frequency of 3 kHz for fast measurements (or 1 kHz for more accurate measurements). We found that adequate measurements could be obtained by acquiring data (12 bit resolution) at only six points per period at times relevant for fitting both exponentials.

The electrical treatment consisted of the application of one or more exponential pulses with a time constant, $\tau_{pulse} = 1.1$ ms, which resulted in $U_{skin,0}$ reaching values which ranged from 50 to 250 V (the range $U_{skin} > 150$ V was seldom reached). During the first 0.3s after a pulse, the response curve exhibits only one significant time constant, and thus in the calculation only one RC-combination

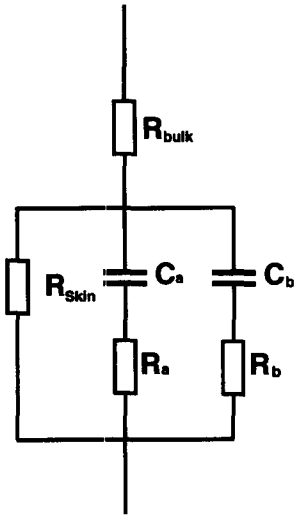

Fig. 3. Circuit model of the measured system. The overall series resistance, R_{bulk}, represents the measuring electrodes and the electrolyte (phosphate-buffered saline pH 7.4 or saline and fluorescent molecules) that filled the chamber compartments (same electrical conditions). The circuit is used to describe the passive electrical properties between the high voltage pulses for a preparation of 0.71 cm^2 of heat stripped stratum corneum. There are three distinguishable pathways for electrical current, leading to different frequency dispersions. A path causing the current at very low frequencies, e.g., dc-current, probably through sweat ducts, hair follicles and aqueous pathways through the lamellar bilayers, is represented by R_{skin}, ranging over two decades for unpulsed skin (20 kΩ to 1 MΩ). There were two distinguishable frequency dispersions in the impedance, believed as a result of capacitances of the lipid layers (sphingolipids, ceramides, C_a, C_b) and saline reservoirs in series. These reservoirs can represent direct pathways, involving the corneocytes (R_b) or tortuous pathways (R_a). The range of the frequency dispersion caused by R_a, C_a is 2–8 kHz and the range caused by the R_b, C_b dispersion is 20–50 kHz. Typical values for the capacitor C_a are 12 nF and 20–200 kΩ for the resistance R_a. For C_b we found about 10 nF and a considerably low resistance $R_b < 1$ kΩ. In case of electroporation, which is believed to create additional aqueous pathways which shunt the entire stratum corneum, this yields a dramatic decrease of R_{skin}.

could be calculated reliably. For this reason, the elements R_b and C_b were not used in seeking quantitative interpretation of the changes in passive electrical properties in this time interval.

The 3 kHz measurements resulted in a time resolution of 10.6 ms if averaging over 16 sampling periods was performed (each period sampling the 6 points). Without averaging the time resolution was 666 μs. Usually we employed averaging in order to reduce noise.

Because of the influence of environmental conditions, such as the temperature, exploratory experiments were first carried out. These measurements revealed that the temperature dependence on R_{skin} for unpulsed preparations was 2.7%/K, which is similar to that of aqueous electrolytes [27]. Because the heating during the pulse was less then 2 K (calculated from power consumption) for the largest pulses, the change of R_{skin} due to heating was negligible compared to the three orders of magnitude change due to 'high-voltage' pulses.

2.6. High voltage pulse regeneration

Pulses were applied either by an ECM-600 (BTX, San Diego, USA) or a GENEPULSER (Bio-Rad, Richmond, CA) by discharging a 25 μF capacitor. Because the pulser discharge time constant τ_{pulse} is the product of the capacitance and the external resistance, and the skin exhibits a large change in the resistance, we fixed the time constant by employing a voltage divider (10 Ω/40 Ω, non-inductive resistances), where the chamber was placed parallel to the 40Ω resistance. The time constant of the 'high-voltage' exponential pulse was always about 1 ms (measured value of 1.1 ms). In order to provide timing for pulsing and measuring, particularly in multiple pulse protocols, an EOP (end of pulse) signal was used. The EOP was generated 3 ms after beginning of the pulse, i.e., the EOP signal appears at $t \approx 3\tau$ after the triggering of the pulse. Our notation therefore refers to the elapsed time after the EOP.

3. Results

3.1. Dynamic resistance during the applied high voltage pulse

The dynamic resistance, R_{dy}, measured during pulsing was found to be considerably smaller than the skin resistance before pulsing. Specifically, within a few microseconds (0.05–2 μs) R_{dy} drops from an initial, pre-pulsed value of 20–200 kΩ (R_{skin}) down to 6000–50 Ω, with the lower values corresponding to the higher voltages (Fig. 4). If the pulse magnitude was increased to $U_{skin,0} > 120$ V,

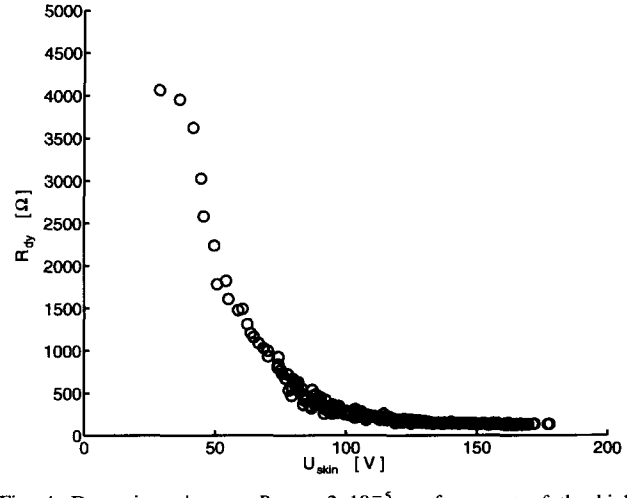

Fig. 4. Dynamic resistance R_{dy} at $2 \cdot 10^{-5}$ s after onset of the high voltage pulse plotted against the voltage across the skin. Below about $U_{skin,0} \approx 40$ V no prompt changes were observed in R_{dy} due to the high voltage pulse. In the region of dramatic changes of the dynamic resistance (pulses of 40–90 V) the changes in the passive electrical properties were usually reversible, whereas in contrast changes for pulses with $U_{skin,0} >$ 110 V seldom recovered. In the range between these values the variability is very high, so that a real limit for reversible altering was not found.

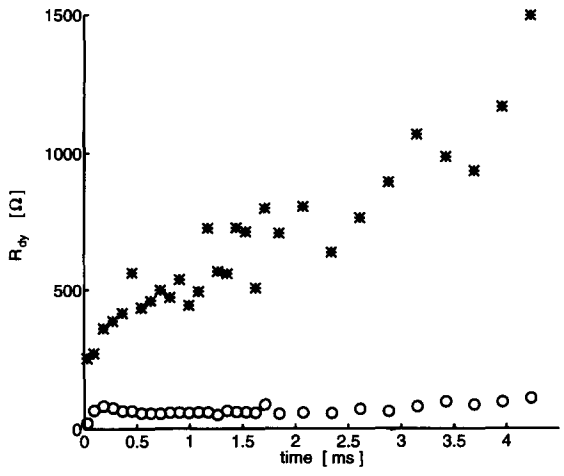

Fig. 5. Time track of the dynamic resistance at high (○, 119 V) and low (∗, 68 V) voltage pulse. The onset of the pulse was at $t = 0$.

R_{dy} nevertheless remained at about 50–100 Ω during the pulse. However, as $U_{skin}(t)$ decays to the range (50–60 V), R_{dy} increases and sometimes reaches more than 1 kΩ for the interval just after the pulse (Fig. 5). If the dynamic resistance falls down to approx. 80 Ω or less, only partial recovery is observed. However if $R_{dy} > 500$ Ω, the response of the skin was found to be almost reversible, as the impedance recovered to within 5% of initial values.

3.2. Voltage across the skin ($U_{skin,0}$)

According to Fig. 6, $U_{skin,0}$ tracks the applied voltage until it reaches about 40–100 V, exhibiting a strong variability on the specimen. Then $U_{skin,0}$ maintains this level, increasing again only when the applied voltage is more then 1000 V.

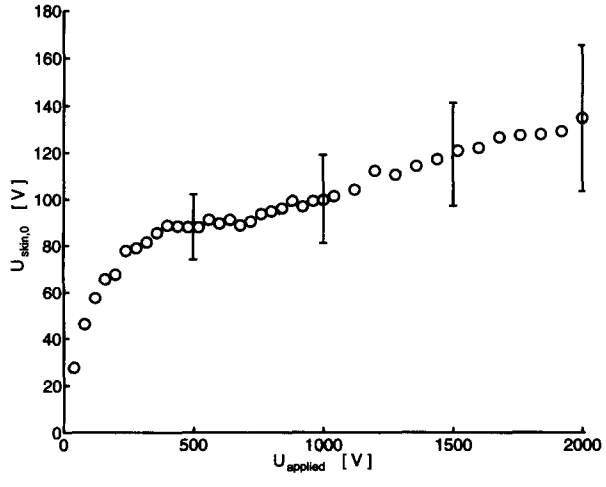

Fig. 6. $U_{skin,0}$ as a function of the voltage applied at the outer electrodes. Higher voltages across the stratum corneum were sometimes reached but not necessarily. For this set of data 9 pieces of skin from two different donors were used.

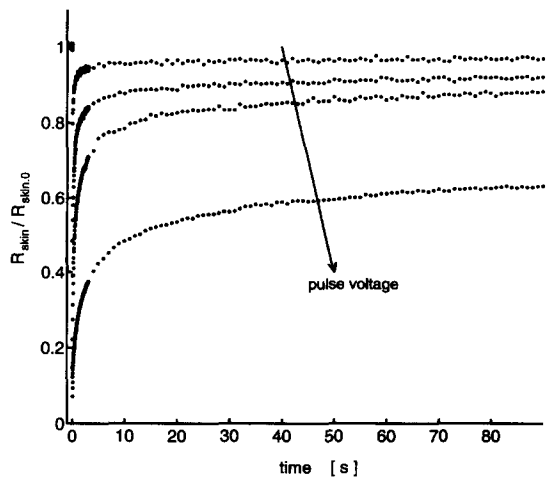

Fig. 7. Typical change of the passive electrical properties (in this case only R_{skin}, i.e., the resistance associated with a purely conductive pathway across the skin) after a single pulse for different voltages across the skin, $U_{skin,0}$. Here $U_{skin,0}$ is calculated using Eq. (1). and extrapolating $U_{skin}(t)$ to t_0, the time of the pulse trigger. The error, estimated by neglecting the charging time of the skin (for the high voltage pulse < 2 μs) and the assumption of an exponential decay of $U_{skin}(t)$ is less than 5%. The values for $U_{skin,0}$ were 71, 88, 105 and 123 V. To facilitate comparisons, the change in resistance was normalized to the prepulse value.

3.3. Electrical recovery of the skin

Following a single 'high-voltage' pulse the recovery of R_{skin} exhibits four phases. Phase one consists of an approx. 20 ms interval immediately after the beginning of the pulse, during which the passive electrical properties cannot be accurately measured because of the noise associated with the switching of the high voltage isolation relay. Although the R_{dy} and R_{skin} characterize the passive electrical behavior, they are not directly comparable, but their large difference can suggest the possibility of this recovery process. At the end of the pulse R_{dy} is order of 50 Ω to 2 kΩ, depending on the pulsing voltage and the skin specimen, whereas the R_{skin} just 20 ms after the end of the pulse is of the order of 2 kΩ to 40 kΩ, which is up to 60% of the prepulsed value of R_{skin}. It then climbs (phase two) to about 40 to 70% of the pre-pulsed value during the next 0 .4–0.8s (Fig. 7). In phase three, the recovery slows significantly, and the recovery progresses in the next 10s to about 60–90% of the pre-pulsed level. Finally, in phase four the recovery process is very long and also different in character. During this phase recovery occurs from 1 min (95%) until a few hours, with incomplete recovery (< 95%) or alteration of the skin due to skin degradation. After very large pulses ($U_{skin,0} > 130$ V) a recovery of more than 50% was usually not observed. In some cases only the first three recovery phases were observed, i.e., the skin was irreversibly altered. Moreover, in some experiments with relatively low voltage pulses ($U_{skin,0} < 80$ V), recovery occurred very rapidly and no more changes were observed

a few seconds after the pulse. We have identified two possible explanations for this 'low voltage' behavior: (1) recovery was complete within this time, or (2) insignificant electroporation occurred.

Three of the time constants are shown in Fig. 8 (the first recovery phase was only suggested from the data, but not measured). From these data it is evident that there is both a trend to longer recovery times and to larger variability (expressed here as standard deviations) of the time constants as larger pulses were used.

Although the most dramatic changes in the skin's behavior involve the transdermal resistance, R_{skin}, the behavior of the other circuit elements were also examined. Here the largest change was found for capacitor C_a, which increases by a factor of up to 4 or 5 in the case of the largest pulses ($U_{skin,0} > 130$ V) (Fig. 9). For smaller pulses ($U_{skin,0} < 90$ V) the change was less than a factor of 1.1. Above $U_{skin,0} \approx 80$ V, the change in C_a was reasonably well-described by a quadratic dependence on $U_{skin,0}$ (Fig. 9). Usually C_a returned to the prepulsed value within 1–3 s. Only after multiple pulses or very strong single pulses (> 130 V) was a permanent increase of C_a observed, and these were as large as 40%.

The elements R_a and C_b showed slightly increased values (5%) 100 ms after the pulse and returned within 300 ms to 800 ms to their initial values. However, persistent changes were usually not observed, and resistance R_b exhibited no significant changes.

3.4. Multiple pulses

After the first pulse in a multiple pulse series the change in R_{skin} depends strongly on the subsequent pulsing history. In order to investigate this case, a protocol involving three pulses with a very long spacing (3 h) was adopted (Fig. 10). The use of such a long interpulse interval was motivated by the observation that the recovery of R_{skin} sometimes occurs over several hours. Because the temperature (25 ± 2°C) and pulse shape remained constant during all the experiments, the percentage of recovery of R_{skin} after the first pulse depends only on the voltage $U_{skin,0}$. In cases of pulses which resulted in $U_{skin,0} < 70$ V, the recovery after the first pulse was sufficient to return R_{skin} within 5% of the initial condition before the next pulse. In contrast, if $U_{skin,0} > 100$ V a full recovery was not observed. As $U_{skin,0}$ was progressively increased, there were larger decreases in R_{skin}, and also less complete recovery. For this reason, at the higher pulse voltages R_{skin} does not fully recover after the first pulse, and therefore the behavior after the second and third pulse was very different from that after the first pulse.

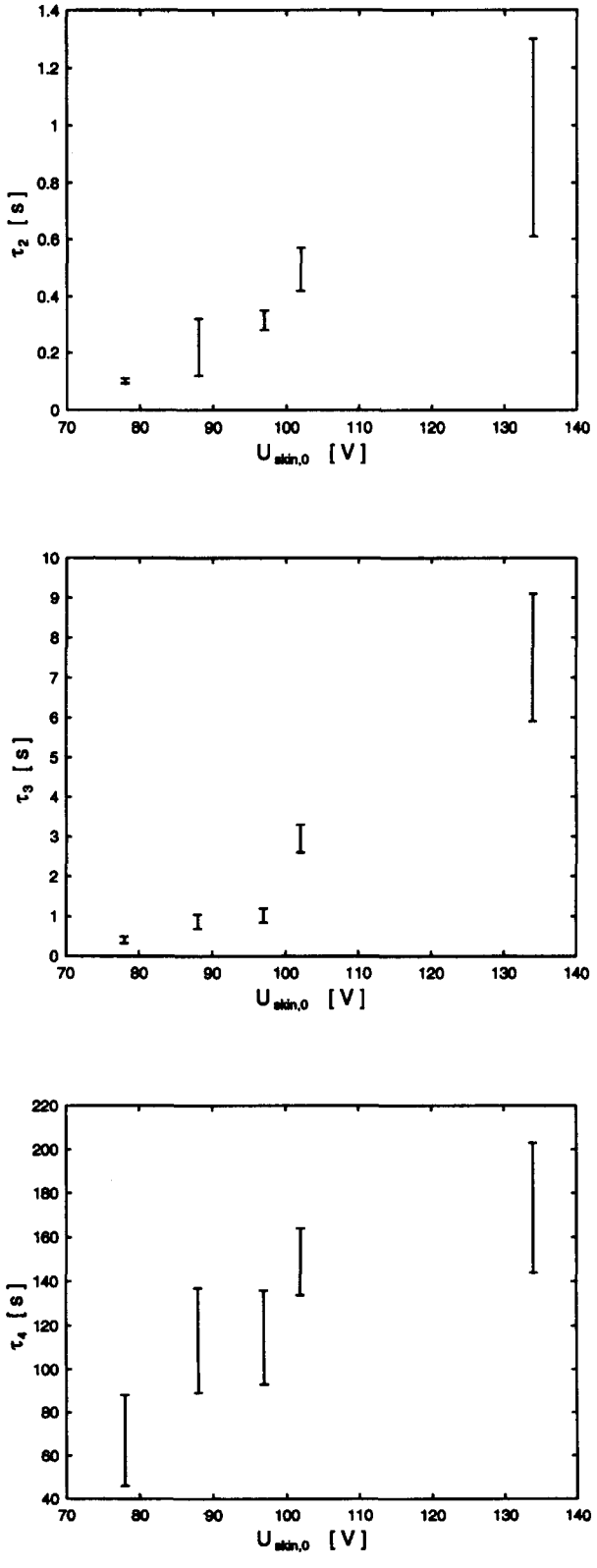

Fig. 8. Time constants of three distinguishable measured recovery processes (without the first one), obtained from 18 different pieces of skin. Only saline was used to bathe the skin (no fluorescent molecule present) in these experiments. Upper panel shows the second time constant versus $U_{skin,0}$ and middle and lower panels, the third and fourth, respectively. For some preparations the recovery after 10 min could also be reasonably fit to an exponential, but for many other preparations the recovery was accompanied by skin degradation, and this prevented a recovery time constant from being identified for the very slow recovery phase.

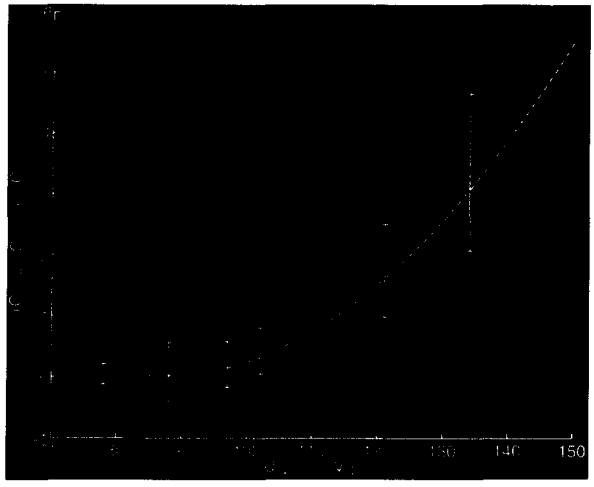

Fig. 9. The change in capacitance C_a, 330 ms after the pulse, in units of the initial (prepulse) value. A quadratic fit for voltages greater than 90 V provides a reasonable description of this change depending on the pulsing voltage. The values presented are average values, as well as the voltage across the skin and the change in capacitance, out of 31 experiments.

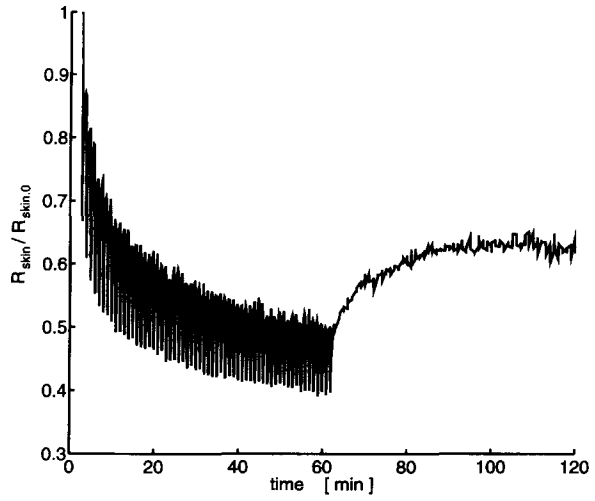

Fig. 11. R_{skin} as a function of time due to multiple pulse protocol. The voltage of the exponential pulses ($\tau \approx 1.1$s) was about $U_{skin,0} = 96$ V, and the spacing 60 s. For better comparison to the stated percentages in the paper R_{skin} is normalized to the prepulsed value.

By decreasing the spacing between the pulses to a minute or less, two effects were found. (1) A quasi steady state occurs for R_{skin} after 15 to 30 pulses, and (2) the minimum value of R_{skin} decreased for shorter spacing. The recovery between the pulses showed a dramatic trend towards little or no recovery for pulse spacing less than 10 s.

Fig. 11 shows an example of change in the most important passive electrical property, the transdermal resistance, R_{skin}, due to a multiple pulse protocol with short (1 min) spacing. Here the recovery is shown within the interval from 330 ms after the end of the previous pulse and to the trigger of the next pulse. It is clear that R_{skin} experiences the largest change because of the first pulse, with partial recovery before the second pulse, and that subsequent pulses cause progressively smaller percentage decreases in R_{skin}. Moreover, the recovery takes place over a longer time, and the degree of recovery progressively decreases as more pulses are applied. Nevertheless, R_{skin} exhibited still two time constants during the recovery process.

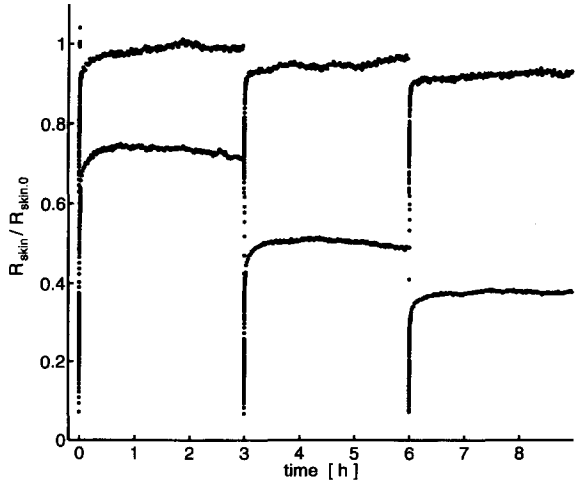

Fig. 10. Recovery of the R_{skin} after widely spaced pulses (time between pulses: 3 h)) using different voltages (upper curve $U_{skin,0} \approx 76$ V, lower curve 104 V). A pulse of $U_{skin,0} \approx 76$ V was followed by nearly full recovery, whereas a voltage of 104 V resulted in only partial recovery.

4. Discussion

As expected qualitatively from the behavior of single bilayer membrane systems [5,28–30], the most important passive electrical property is R_{skin}. In single bilayer membrane systems, short pulses that cause the transmembrane voltage to reach 0.5 to 1 V result in large, very rapid decreases in the membrane resistance, R_m [18,21,31,32]. This dramatic electrical behavior can be understood quantitatively using a transient aqueous pore model [19,23,31,33,34]. With this in mind, it is expected that application of one or more 'high-voltage' pulses to skin preparations might result in large, rapid changes in R_{skin}. Again based on experiments with single bilayer membrane systems, particularly cells, varying degrees of recovery over a much longer time scale might be expected. As noted previously [8], and confirmed here, because the SC contains approx. 100 bilayer membranes in series, the simple prediction from single bilayer electroporation studies is that large and very rapid changes in R_{skin} should appear for pulses which cause $U_{skin,0}$ to reach 50–100 V. The present study supports this expectation. Here the decrease in R_{skin} was found to be very rapid, and also sensitive to value of $U_{skin,0}$ achieved because of 'high-voltage' pulsing. Specifically, here $U_{skin,0} \approx 50$–150 V was achieved by

individual pulses in single or multiple pulse protocols. The rapid (< 2 μs) onset and the change of R_{skin} by three orders of magnitude is also interpreted here as being consistent with electroporation occurring within the SC.

Although the associated changes in molecular transport is not the topic of the present study, we separately found that R_{skin} changes correlate well with the time related transport of calcein ($M = 623$, $z = -4$) caused by a series of 'high-voltage' pulses (unpublished observation). Qualitatively, this correlation is generally expected, because the transport of small ions through aqueous pathways will be indicative of pathways available for the transport of molecules that can 'fit' into these pathways. In contrast, the pathways for ac current flow, i.e., involving voltage changes with higher frequency components, can involve displacement currents through capacitance elements. However, there is no actual transport of ions across these capacitances. For this reason, the capacitance-based pathways do not contribute to ionic or molecular transport across the skin.

The possibility of using electrical measurements as indicators for molecular transport was a significant motivation for undertaking the present study. Indeed, as shown here, electrical behavior measurements can be made rapidly (of order ms) where molecular transport measurements require transport of the participating molecules to a measurement site at least several mm from the surface of the skin, so that even a miniaturized flow-through permeation chamber system has a minimum response time of about 1s [35].

The time constant for charging and discharging the skin preparation via the external pathways through the chamber and electrodes is of the order of 20 to 100 μs. The time constant associated with discharging the skin through internal (perforating) aqueous pathways changes tremendously from the unpulsed value

$$\tau_{int,\,unpulsed} = R_{skin,\,unpulsed} \cdot C_{skin,\,unpulsed} \approx 1\,ms$$

where C_{skin} is the lumped capacity of the skin, down to

$$\tau_{int,\,pulsed} \approx R_{skin,\,pulsed} \cdot C_{skin,\,pulsed} \approx 500\ \Omega \cdot 10\ \text{nF} \approx 5\ \mu\text{s}.$$

This means that the transport of small ions is expected to closely follow the applied pulse, which has a time constant $\tau_{pulse} \approx 1.1$ ms $>> \tau_{int}$. On the longer time scale, with which molecular transport is measured (1–30 s), this means that electric field-driven transport of charge ceases as soon as the pulse stops. Some aspects of this electrical behavior, particularly the change in the effective time constant for the internal pathway discharge, have previously been found for electroporated artificial planar bilayer membranes [18,31], with the membrane resistance (here replaced by R_{skin}) interpreted as due almost entirely to small ion transport through transient aqueous pores [19,23].

The present study found that there is considerable variability between different skin preparations as well as in the onset behavior and the recovery of the impedance. Further a strong dependence on the magnitude and number of pulses used was observed. Keeping in mind the overall motivation of altering molecular transport across skin and the fact that it would be difficult to rapidly optimize transport for individual skin sites by using molecular transport itself, determination of the passive electrical properties of pulsed skin may provide a rapid, initial optimization for a molecular transport protocol. This is expected to include both determination of the pulsing needed to cause electroporation, and determination of the extent of the skin barrier recovery. Although for our pulsing protocols there is a characteristic voltage range ($U_{skin,0} = 80$–100 V), where a transition from reversible to irreversible changes in the stratum corneum was found, the recovery of the skin varies strongly between individual skin preparations and the applied pulsing protocol. With this in mind, it may be helpful to use essentially continuous electrical measurements at very low voltages (e.g., $U_{skin}(t) = 100$ mV) to provide an indication of the condition of the skin. Such an approach may allow, for example, avoidance of irreversible skin barrier changes.

Based on what is believed to occur for transient aqueous pores in electroporated single bilayer membranes, the shrinkage and/or disappearance of new aqueous pathways caused by pulsing may be responsible for the recovery processes. Because the time for closing aqueous pathways ('pores') in single bilayer systems is distributed over an extremely wide time range (10^{-4} s to 10^{3} s), it is not surprising that here the recovery ranges from a few ms up to several hours. In the case of very large pulses ($U_{skin,0} >$ 130 V), the decrease of the skin dynamic resistance was extremely large (sometimes reaching $R_{dy} \approx 50\ \Omega$ during one pulse) and the subsequent recovery incomplete.

Interestingly, the initial recovery appears to be similar for all pulses. This possibility is suggested by the observation here that the increase of R_{skin} during the first 100 ms after the pulse is approximately the same as for lower voltage pulses. This suggests that the initial process of recovery is similar, independent of the individual properties of the skin, and on the pulsing voltage. In the case of recovery after a very large voltage pulse, a maximum of recovery level of about 50% was found for R_{skin}, which means that for these large pulses the skin seems incapable of closing some of the newly created aqueous pathways. Thus one possibility is that for small pulses a number of small aqueous pathways are created, and all of these pathways shrink and then close. However, for large pulses more pathways may be created, such that initially all pathways shrink, but subsequently some pathways cannot close completely and are responsible for the essentially permanent change in R_{skin}. If the present understanding of single bilayer membrane electroporation is directly relevant, then larger pulses are expected to create more pores, but actually fewer large pores [23]. The question of the

size distribution of the electrically created aqueous pathways is important, but cannot be elucidated by electrical measurements alone, as ionic conduction is dominated by the smallest, most mobile ionic species. Only if unusual (non-physiological) electrolytes were used, viz. electrolytes with larger ions and perhaps multivalent ions, could additional insight into the pathway size be obtained, and in this case there is the danger that non-physiologic media may itself cause changes to the SC. With this in mind, a better understanding of the size distribution of the new pathways will depend on making simultaneous molecular and electrical transport measurements, which is beyond the scope of the present study.

The skin capacitance changes are not nearly as pronounced, and the changes that do occur have a relatively short lifetime. For example, the changes in the capacitance C_a (up to 5 times) persisted for only about 1 s. This is in marked contrast to the case of single bilayer membrane electroporation, for which small (< 1 or 2%) capacitance changes occur [23,32]. Unlike single bilayer membrane systems, however, the complex SC has the possibility of admitting water through temporary pathways that may become temporarily trapped. Qualitatively, because of the large dielectric constant of water ($\epsilon_w = 80$) compared to the dielectric constant of lipid ($\epsilon_l = 2$), temporary addition of free water should increase the capacitance of the SC. Even the fully hydrated SC is believed to ordinarily contain very little water in the regions between adjacent bilayers. It has, for example, been suggested that there is ordinarily only about one water molecule per head group of the ceramide head groups of the lipids. This small water content must mean that it is energetically unfavorable for more water to exist in these regions, because there is an ample supply of water by passive permeation. The usual hydration process that allows corneocytes (most surrounded by bilayer membranes) to take up large amounts of water, and then swell, suggests that the water flux through the bilayers is possible. Additional water entry into the space between bilayers is favored during the short time that a pulse exists. If perforating aqueous pathways form (our present hypothesis), then a rapid supply of water into the inter-bilayer regions should be possible. As the pulse decays, water could then be driven back out of the SC, with the kinetics controlled by the forces that favor a low water content and the flow resistance in the small spaces between the bilayers and within the aqueous pathways across the SC.

Another possibility for the temporarily increased C_a is that during the recovery phase a completely perforating aqueous pathway is first closed by one, and then a small number of SC lipid bilayers, and this is equivalent to 'switching in' capacitance which was previously shunted. An inability to close some of the pores may be an explanation for the resident changes in the C_a after many or very large pulses.

For the remaining circuit elements (R_a, C_b, R_b) changes due to high voltage pulses were qualitatively apparent, but too small for quantitative interpretation. Thus, these parameters are only used to provide a more complete description of low voltage behavior.

5. Conclusions

An understanding of the behavior of the SC to 'high-voltage' pulses is not only of fundamental interest, but it is also relevant to finding a conveniently measured parameter that can serve as an indicator of changes in the skin barrier. For potential applications the main interest is the control of molecular transport across the skin, which involves both changes in the permeability and in the local driving forces across the skin. Although not strictly necessary, a reversible process is viewed as desirable. By using electrical measurements to determine electrical changes of the stratum corneum either during pulsing (R_{dy}) or recovery behavior of the SC (R_{skin}), the pulsing protocol can be optimized for both small ion transport and recovery.

From the change in R_{skin} and R_{dy} due to $U_{skin,0}$ we conclude that there are three outcomes that result from electrical pulsing: (1) no measurable indication of changes in R_{skin} if R_{dy} is not below 500 Ω, which is interpreted as electroporation not occurring, and therefore the skin is unaltered, (2) reversible electroporation, where new aqueous pathways are created, and recovery of R_{skin} takes place in seconds to minutes (R_{dy} between 150 Ω and 500 Ω), and (3) irreversible electroporation, in which aqueous pathway recovery is incomplete ($R_{dy} < 150$ Ω).

The electrical measurements described here provide basic information as to how the SC responds to 'high-voltage' pulses. These measurements may provide sufficiently rapid monitoring capability for reversible electroporation conditions.

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