Effects of Application Voltage and Cathode and Anode Positions at Electroporation on the *in Vitro* Permeation of Benzoic Acid through Hairless Rat Skin[1)]

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The enhancing effect of electroporation on the *in vitro* skin permeation of benzoate was evaluated. Needle and ring electrodes made of Ag/AgCl were connected to an electrical power source, which produced exponentially decaying pulses. The needle electrode was kept in contact with the skin surface, and the ring electrode was positioned either on or under the skin. The electrical pulse was applied to abdominal hairless rat skin at 150—600 V every minute from 4 to 6 h during the 10-h permeation experiment. Skin permeation of benzoate was promoted by electroporation and the effect was increased by application of a higher voltage. No immediate recovery to the control flux, however, was observed for high voltage groups after turning off the voltage application. When the cathode and anode were separated by the skin membrane by setting in the epidermal and dermal sides, respectively, an iontophoretic effect may also play a role in benzoate flux. These results indicated that the drug permeation by electroporation is the result of passive diffusion and an iontophoretic effect as well as the electroporation effect.

Key words electroporation; skin permeation; benzoic acid; electrode position; application voltage

Improvement in skin permeation rate of drugs is necessary to effectively use the skin surface as a site for administration of many drugs. There are two methods for enhancement of skin permeation. The first is to increase thermodynamic activity or electrochemical potential of a drug in the formulation; for example chemical modification to a prodrug with higher thermodynamic activity than the parent drug and application of iontophoresis to weak acid or basic drugs. The other is related to enlargement or creation of permeation pathways in the skin barrier, the stratum corneum; use of chemical enhancers may be included in this category. In addition, electroporation is a good example of this latter mechanism, because it causes pore formation in biological membranes.[2)] Electroporation has been widely used for introducing DNA and RNA into cells and biological tissues, since the original model was developed by Neuman *et al*.[3)] and Zimmermann *et al*.[4)] Recently, this electroporation technology was utilized to increase transdermal drug delivery.[5—8)] We also reported the effect of electroporation on the skin permeation of mannitol, a nonionized model material.[9)] In the report we compared the effect by electroporation with iontophoresis. In the present study, sodium benzoate was used as a model ionized material. The effect of applied potential was determined as a continuation of the mannitol study. The possibility of the effect of iontophoresis was also evaluated on the skin permeation of benzoate at electroporation.

Experimental

Materials Sodium benzoate was obtained from Wako Pure Chemicals (Osaka, Japan). Other reagents were of analytical grade and were used without further purification.

Needle type Ag/AgCl electrodes (1.0 mm in diameter and 3 cm in length) and ring type Ag/AgCl electrodes (0.04 mm in thickness) were prepared by silver plate (Murata Yohaku, Tokyo, Japan) in our laboratory. The tip of the needle electrode was bent to prevent damage of the skin barrier.

Skin Permeation Experiment Male hairless rats (WBN/ILA Ht), 210—230 g, were supplied either by Life Science Research Center, Josai University (Sakado, Saitama, Japan), or Ishikawa Experimental Laboratory (Fukaya, Saitama, Japan). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the abdominal skin was excised. After trimming, the fresh skin piece was mounted in a vertical Franz type diffusion cell with an effective diffusion area of 3.14 cm^2, with a water jacket connected to a water bath maintained at 37 °C.[10)] Sodium benzoate at a concentration of 30 mg/ml in physiological saline (3.0 ml) was applied to skin, with just saline (17.0 ml) on the dermal side. The permeation experiment was performed for 10 h. The receiver side was stirred at 1200 rpm with a star head-magnetic bar and a magnetic stirrer (Multistirrer, Scinics, Tokyo). Skin permeation of benzoic acid was followed by periodic sampling of 0.5 ml from the receiver solution, and then the same volume of fresh saline was added to keep the volume constant. The drug concentration in each sample was assayed by HPLC to determine the flux through the skin at each sampling period. The animal experiments were performed in accordance with the guidelines of Life Science Research Center, Josai University.

One needle electrode was always set on the skin surface. A ring electrode was positioned either on the skin surface or the dermal side. The needle electrode was either the cathode or anode with the ring electrode acting as the respective opposite terminal. The diffusion cell and the position and size of the electrodes and are shown schematically in Fig. 1. These electrodes were connected to a Gene Pulser® (Bio-Rad, Hercules, CA, U.S.A.), commonly used for electroporating bacterial and other cell membranes. The power source delivers exponentially decaying pulses. Capacity of the electroporation apparatus was set at 1 μF, and 120 pulses of 150, 300, 450 or 600 V were generated for 2 h (1 pulse/min).

Assay Each sample was mixed with the same volume of acetonitrile containing 10 μg/ml *p*-ethyl benzoic acid as an internal standard. After centrifugation, the mixed solution was injected into an HPLC apparatus composed of a pump (LC-10AS, Shimadzu, Kyoto, Japan), UV detector (SPD-10A, Shimadzu), integrator (C-R5A, Shimadzu), system controller (SCL-10A, Shimadzu), auto injector (SIL-10AXL, Shimadzu) and a reverse phase column (Lichrospher® 100 RP-18(e), 4 mm×250 mm, Kanto Kagaku, Tokyo). Flow rate was 1.0 ml/min, mobile phase was acetonitrile : 0.05 M phosphate buffer (1 : 1) and UV wavelength was 230 nm.

Results

Figure 2 shows the time course of benzoate flux through excised hairless rat skin. Electroporation was applied to the skin for 4 to 6 h. Figure 2a shows the results obtained with the needle electrode as the cathode and ring electrode as the anode, and Fig. 2b shows those under the reverse conditions. An almost constant (steady-state) flux was observed from

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about 2 h without voltage application (control). When the voltage (150—600 V) was applied every minute, flux increased almost linearly with time in both cases (Figs. 2a and b). After turning off the voltage application, however, the flux was not recovered to the control value especially following application at 450 or 600 V. In contrast, the flux showed a tendency to return to the control value after electroporation at a low voltage (150 V). The maximum flux was observed from 5.67 to 6 h. The profiles in Fig. 2a were similar to those in Fig. 2b.

The ratio of the maximum flux (each group/control group) was then plotted against voltage difference from the needle to ring electrode (Fig. 3). Regardless of whether the needle electrode was the cathode or anode, the flux increased with increases in the voltage applied. The increase was almost completely linear when the needle electrode was used as the anode, whereas a slightly convex curve was obtained when the needle electrode was the cathode. Each flux was the sum of the electrically assisted transport and passive diffusion.

The ring electrode was set under the skin, whereas the needle electrode was applied to the skin surface. Figure 4 shows the time course of benzoate flux through excised hairless rat skin. Similarly to the experiments shown in Fig. 2, 150 to 600 V was applied using the needle electrode as the cathode and ring electrode as the anode (Fig. 4a) or *vise versa* (Fig. 4b). The permeation fluxes during electroporation shown in Fig. 4b were similar to those shown in Figs. 2a and b. In contrast, the fluxes during electroporation in Fig. 4a were higher than the others, when the skin permeation was compared among the same application voltage.

The ratio of the maximum flux was again plotted against voltage difference from the needle to ring electrode (Fig. 5). The flux profile when the needle electrode was used as the anode was almost the same as that shown in Fig. 3. Interestingly, a higher flux was observed when the needle electrode was set as the cathode.

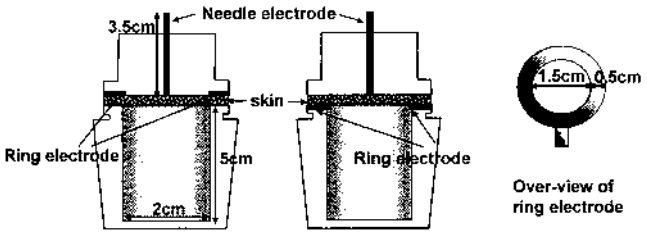

Fig. 1. Schematic Illustration of the Diffusion Cell and the Position and Size of the Electrodes

Discussion

Electroporation is a technique to promote gene introduction into cells and biological tissues.[3,4] The effect of electroporation was reported to be reversible.[11] In the present skin permeation study, however, the effect of electroporation on the skin permeation of benzoate was not reversible, especially at high voltages. Voltage application probably causes the formation of tiny pores in the primary skin barrier, the stratum corneum. Ceramides, primary lipids, are tightly and rigidly packed in the stratum corneum compared to lecithin in other biological membranes. Thus, it may be very difficult for pores produced in the stratum corneum by electroporation to immediately close again, which may explain the observed irreversibility of the electroporation effect.

The enhancement ratio at the application of 600 V was calculated to 3- to 3.5-fold as compared to the control (Fig. 3). Electroporation enlarges the permeation pathway in the skin barrier to enhance the skin permeation of drugs. When electroporation is applied to skin, the resulting flux may be due to the sum of electrically assisted flux and original passive diffusion, the former of which contains passive diffusion through newly created pore pathway. Therefore, the original pathway for the passive diffusion may be decreased with the pore creation by electroporation.

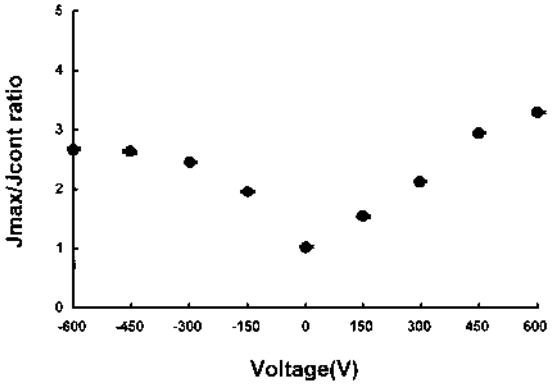

Fig. 3. Relationship between Enhancement Ratio and Electroporation Application Voltage with Both Electrodes on the Skin Surface

J_{max} is the maximum flux observed from 5.67 to 6 h, and J_{cont} is steady-state flux without of voltage application. Each point represents the mean±S.E. of three experiments.

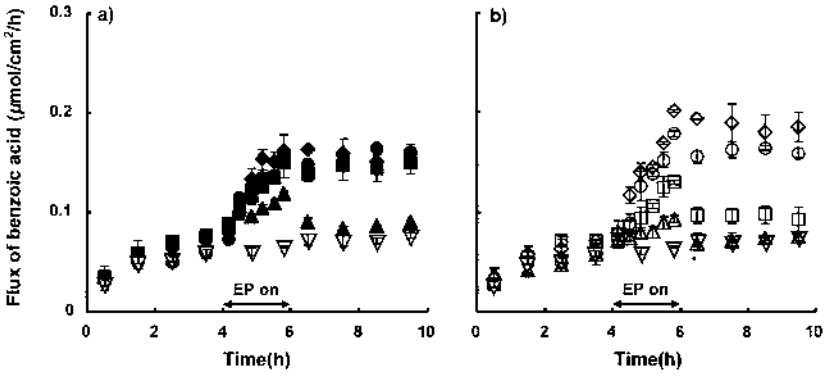

Fig. 2. Effect of Application Voltage on the Skin Permeation of Benzoate with Both Electrodes on the Skin Surface

▽, 0 V (control); ▲ and △, 150 V; ■ and □, 300 V; ● and ○, 450 V; ◆ and ◇, 600 V. Closed and open symbols represent that needle electrode was cathode (a) and anode (b), respectively. Each point represents the mean±S.E. of three experiments.

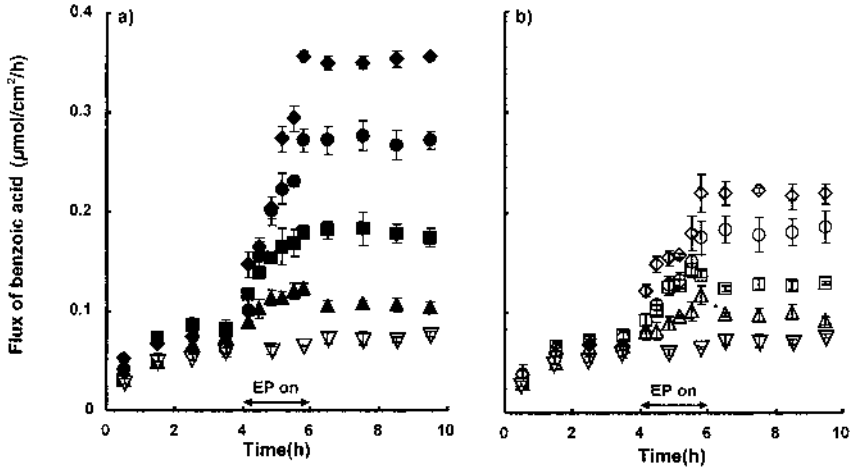

Fig. 4. Effect of Application Voltage on the Skin Permeation of Benzoate with the Two Electrodes Separated by the Skin

Symbols are the same as in Fig. 2. Each point represents the mean±S.E. of three experiments.

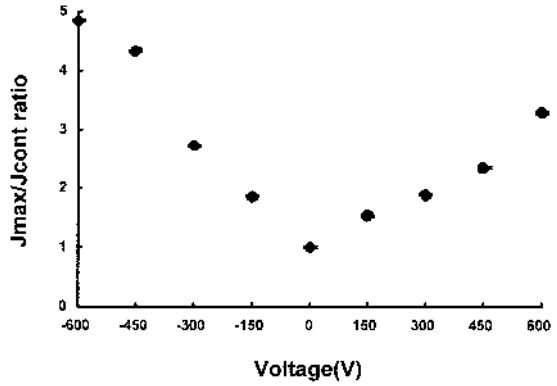

Fig. 5. Relationship between Enhancement Ratio and Electroporation Application Voltage with the Two Electrodes Separated by the Skin

Each point represents the mean±S.E. of three experiments.

Benzoic acid is an anionic drug, so there may also be an iontophoretic effect when the cathode is applied to the donor (stratum corneum) side with the anode in the receiver (dermis) side. Thus, the electrically assisted flux may contain that by the iontophoretic effect. The differences in flux between Figs. 2a, 2b, 4b and Fig. 4a may have been due to the iontophoretic effect. This was lower than the inherent electroporation effect under the present conditions. However, flux-decreasing effect by iontophoresis was not observed when anode was applied to the donor side with the cathode in the receiver (see Figs. 2b and 4b), probably because the primary mechanism of benzoate permeation through skin without electroporation is passive diffusion of its unionized form, not of ionized form.

In conclusion, the *in vitro* skin permeation of benzoate was promoted by electroporation and the effect was increased by application of higher voltage. No immediate recovery to the control level, however, was observed following application of a high voltage. When the cathode and anode were separated by the skin membrane by setting in the epidermal and dermal sides, respectively, an iontophoretic effect may also be involved in benzoate flux. Drug permeation during electroporation is a result of passive diffusion and the iontophoretic effect as well as the electroporation effect.

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References and Notes

1) Part of this work was presented at the Millennial World Congress of Pharmaceutical Sciences, San Francisco, April 2000.
2) Rosenheck K., Lindner P., Pecht I., *J. Membrane Biol*., **20**, 1—12 (1975).
3) Neumann E., Rosenheck K., *J. Membrane Biol*., **10**, 279—290 (1972).
4) Zimmermann U., Schulz J., Pilwat G., *Biophys. J*., **13**, 1005—1013 (1973).
5) Jadoul A., Lecounturier N., Mesens J., Caers W., Preat V., *J. Controlled Release*, **54**, 265—272 (1998).
6) Lombry C., Dujardin N., Preat V., *Pharm. Res*, **17**, 32—37 (2000).
7) Sharma A., Kara M., Smith F. R., Krishnan T. R., *J. Pharm. Sci*., **89**, 528—544 (2000).
8) Chang S., Hofmann G. A., Zhange L., Deffos L. J., Banga A. K., *J. Controlled Release*, **66**, 127—133 (2000).
9) Mori K., Watanabe T., Hasegawa T., Sato S., Sugibayashi K., Morimoto Y., *Drug Delivery System*, **14**, 485—490 (1999).
10) Yukawa J., Sugibayashi K., Morimoto Y., *Yakuzaigaku*, **49**, 254—262 (1989).
11) Donald C. C., Thomas S. R., *Biophys. J*., **58**, 1—12 (1990).