Effects of Calcium Modulation on Percutaneous Absorption of a Model Drug

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Purpose. To investigate the effect of modulation of the calcium gradient in the skin on the percutaneous absorption of nicotinic acid (NA).

Methods. A skin model with an altered calcium gradient was produced by pretreatment with A23187 (25 μg/ml). The immunohistochemistry was used to evaluate the change in phosphoprotein Elk-1. ATR-FTIR spectroscopy was employed to investigate the changes in lipid conformation in the skin. The permeation profiles of the model drug were studied and the distribution profile of the model drug in the skin layer was evaluated using the cryostat micro-sectioning method.

Results. The immunohistochemistry demonstrated that modulation of the calcium gradient increased phosphorylated Elk-1 in the dermis. The FTIR study showed a shift of the asymmetric stretch peak, implying an increase in lipid fluidity in the epidermis. The amount of NA permeated through the A23187-pretreated skin was significantly lower than that of the control (2.2714 ± 0.6040 vs. 3.1895 ± 0.2456 $\mu g/cm^2/h$). The Cryostat micro-sectioning study showed that there are concentration gradients of the model drug across the skin layers with a higher gradient in the control than in the A23187-pretreated skin.

Conclusions. The alteration in the calcium gradient significantly decreased the permeation rate of a hydrophilic drug, such as nicotinic acid.

KEY WORDS: A23187; calcium; lipid bilayer; nicotinic acid; permeation.

INTRODUCTION

Calcium is a divalent ion vital to many biologic processes. In the skin, calcium has an important role in regulating epidermal keratinocyte physiology and maintaining skin structural integrity. Low calcium concentrations (0.05 to 0.1 mM) stimulate keratinocyte proliferation, whereas high concentrations (>1.0 mM) lead to stratification and differentiation (1). Ion-capture cytochemistry has demonstrated the presence of a calcium gradient in the skin membrane (2). Extracellular calcium is low in the basal and spinous layers, and gradually increases from the inner to outer layers of the stratum granulosum (3). The cytosolic calcium content is high within the outer nucleated layers of the epidermis. A Ca^{2+} concentration gradient appears to regulate growth and differentiation of epidermal cells, and thereby maintain an effective permeability barrier.

Disruption of the permeability barrier results in a marked decrease in the quantity of calcium in the outer nucleated epidermis and a disappearance of the calcium gradient (4). A change in the calcium gradient may lead to increased calcium signaling, which may subsequently induce the activation of various processes, such as increased synthesis of cell components or messengers to the inflammatory reactions. Skin diseases, in which the normal calcium gradient appears to be altered, such as psoriasis and acne, are characterized by an impaired functioning of the permeability barrier (5,6). In this work, alteration of calcium gradient in the skin was produced to simulate the pathologic conditions of skin diseases.

A23187, a lipid soluble carboxylic acid antibiotic, is known to transport calcium across various biologic membranes (7). Studies performed on a variety of tissues have revealed that the effects of A23187 on cellular calcium distribution are very complex. They are dependent on the cell type, ionophore concentration and extracellular calcium concentration. It has been suggested that higher concentrations of ionophore must be used to produce the changes in calcium gradient in tissue slices than in isolated cells (8). A23187 also is known to accelerate mucin secretion by a noncompound exocytotic pathway (9). These strong associations can lead to cross-linking with proteins and membrane conformational changes. Many of the activities of calcium involve interactions with proteins to which calcium binds tightly and exerts a stabilizing, activating or modulating effect. Elk-1 phosphorylated by Ca^{2+} dependent mitogen-activated protein (MAP) kinase signaling cascades (10) was monitored as a marker for the alteration of the calcium gradient in the skin.

The objective of this study was to investigate the effect of an altered calcium gradient in the skin on the permeation profiles of the model drug. The major hypothesis was that pretreatment of the skin with A23187 decreases the permeation rate of a highly ionized drug through the skin due to interaction between ionized compounds and calcium accumulated in the intercellular compartment. A skin model with an altered calcium gradient was produced by treatment of the skin with the cationic ionophore, A23187. Immunohistochemistry was used to evaluate the change in calcium gradient in the skin upon exposure to A23187 by monitoring phosphorylated Elk-1 (pElk-1) immunoreactivity. Nicotinic acid (NA), a highly ionized drug at pH 7.4, was used as a model drug. The changes in the lipid conformations of the skin membrane upon exposure to A23187 were studied using attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy (11,12,13). The distribution of drug in the skin during the permeation was evaluated using the cryostat microsectioning technique.

MATERIALS AND METHODS

Materials

Radiolabeled (carboxy-^{14}C) NA (specific activity: 47 mCi per mmol) and unlabeled NA were obtained from Sigma (St. Louis, MO, USA). The free acid form of A23187 was ob-

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ABBREVIATIONS: NA, nicotinic acid; HHBSS, HEPES buffered Hanks balanced salt solution; PBS, Phosphate buffered saline; pElk-1, phosphorylated Elk-1; ATR-FTIR, Attenuated total reflectance Fourier Transform spectroscopy; MAP kinases, mitogen-activated protein kinases HBSS.

tained from Calbiochem (La Jolla, CA, USA). Hanks balanced salt solution (HBSS) was obtained from GIBO BRL (Long Island, NY, USA). Phospho-Elk-1 antiserum was obtained from Cell Signaling Technology (Beverly, MA, USA). All other reagents and solvents were of analytical grade.

Methods

Preparation of Skin Samples

Dorsal skin was freshly excised from male hairless rats (Sprague-Dawley, 200-250 g) (Charles-River Inc., Wilmington, MA, USA) euthanized with a lethal dose of sodium pentobarbital (50 mg/kg, i.p.). Any adhering fat and subcutaneous tissue were carefully removed from the excised skin without damaging the skin. Skin samples were rinsed with HEPES buffered Hanks balanced salt solution (HHBSS, pH 7.4) and treated with various concentrations (1–25 μg/ml) of A23187 at room temperature for 1 h The control samples were treated with HHBSS. The pretreatment step was carried out under minimal light exposure, since A23187 is light sensitive.

Study of Skin Morphology

Skin samples were treated with A23187 for 1 h, and tissue pages (2–3 mm) were transferred into 4% paraformaldehyde in 0.1 M PBS at 4°C for 10 min. Using a cryostat microtome, frozen samples were cut longitudinally into sections with 20 μm thickness and each section was mounted on a chrom-alum/gelatin-coated slide. After drying sections at room temperature, a routine hematoxylin and eosin staining procedure (14,15) was performed to examine the skin morphology in each section.

Immunohistochemical Study

For the immunohistochemistry with pElk-1 antiserum (16), another set of tissue pages was transferred into 4% paraformaldehyde in 0.1 M PBS at 4°C for 10 min. Tissue pages were postfixed in 10%sucrose/4% paraformaldehyde at 4°C for 2 h and then placed in 20% sucrose/PBS at 4°C overnight. Using a cryostat microtome, the frozen samples were cut longitudinally into sections with 40 μm thickness and all the sections were collected in 0.1% sodium azide/PBS. Sixteen sections per group were randomly collected and processed for the immunohistochemistry. Phospho-Elk-1 antiserum (Cell Signaling Technology, 1:6000) is known to interact only with the phosphorylated Elk-1 protein at the site of amino acid Ser383 (17). Sections were incubated with the antiserum at 4°C for 20 h on a shaker. Sections were then incubated in goat anti-rabbit secondary antiserum (Vector Labs, Burlingame, CA, USA) for 1 h followed by avidin-biotin-peroxidase reagents (Elite Vectastain Kit, Vector Labs) at room temperature for 1 h Diaminobenzidine (Sigma) was used as the chromagen. Throughout the immunohistochemical study, all the

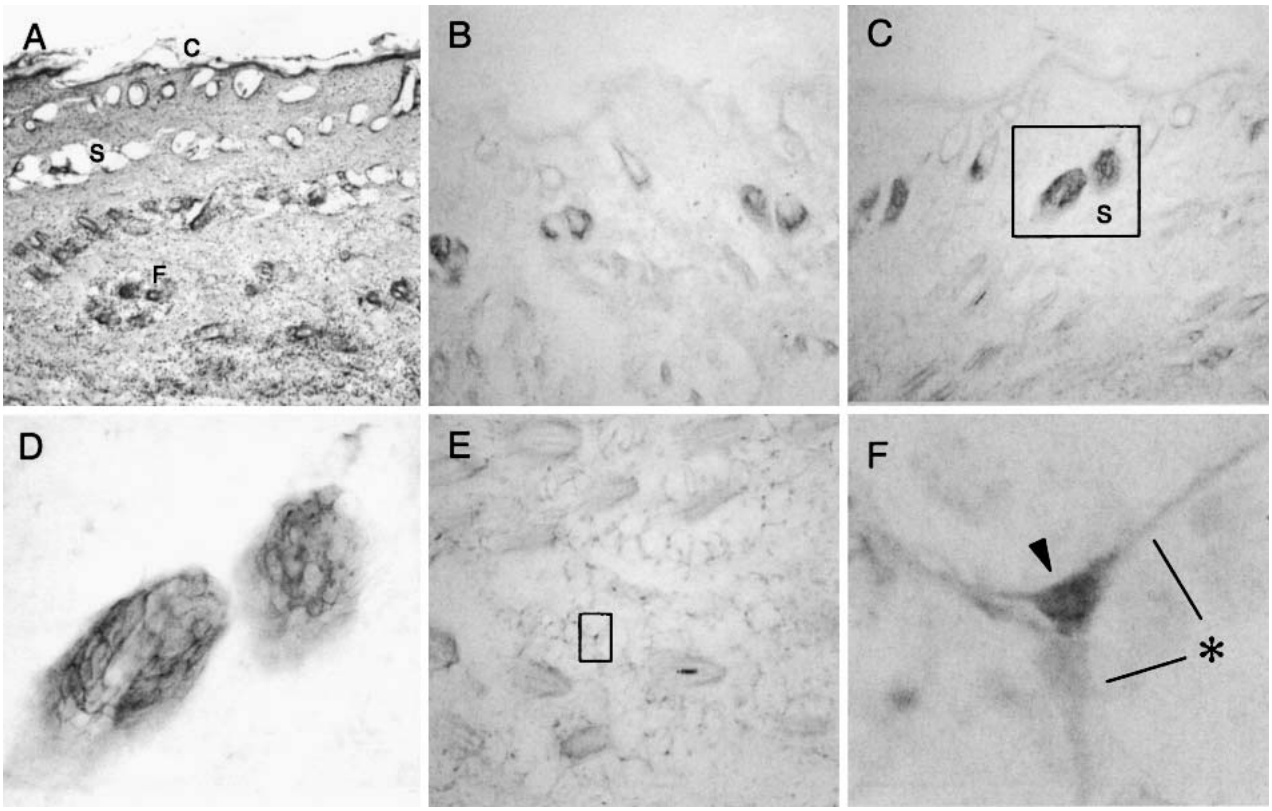

Fig. 1. Skin morphology and pElk-1 immunoreactivity in rat dorsal skin A. The morphology of the skin containing sebaceous glands (S), hair follicles (F) and stratum corneum (C) using the hematoxylin and eosin stain method (×10). B–F. Effects of A23187 (C–F) on the induction of pElk-1 immunoreactivity in the epidermis and dermis as compared with the control (B) (×10). D. Higher magnification of the sebaceous glands taken from boxed area in panel C (×20). E. Reticular layer in the dermis shows increased pElk-1 immunoreactivity (×20). F. Higher magnification of cells in the reticular layer in boxed area illustrated in panel E. Note that prominent pElk-1 immunoreactivity is confined to the nucleus (arrowhead) than cytoplasm (*) (×40).

sections were run simultaneously under the same conditions. Sections were mounted on chrom-alum/gelatin-coated slides, coverslipped in Permount, and photographed with a Nikon E800 photomicroscope. Level of the pElk-1 immunoreactivity in the tissue sections was qualified using NIH Image 1.62 software.

ATR-FTIR Spectroscopic Study

The changes in the lipid conformations of the skin resulting from calcium modulation were studied using ATR-FTIR spectroscopy. A Nicolet FTIR spectrophotometer equipped with a horizontally ATR kit (Nicolet, Madison, WI, USA) was used. ATR-FTIR spectra of rat dorsal skin samples, either pretreated with A23187 or the control samples, were examined in the range of 4000–450 cm^{-1}. The spectra of the epidermis-exposed skin were deconvoluted in the region of 3000–800 cm^{-1} with Omnic software program to resolve the spectrum. All spectra reported were the average of 50 scans with a resolution of 4 cm^{-1}. Measurements were made at ambient laboratory conditions (relative humidity 50–60%; temperature 25 ± 1°C).

In Vitro Permeation Study

Skin samples pretreated with various concentrations (1–25 μg/ml) of A23187 were sandwiched between the half-cells of a Franz permeation cell (Permegear, Somerville, NJ, USA) with a mucosal surface exposed to a receptor half-cell filled with HHBSS (pH 7.4). The volume of the receptor cell was 5 ml and the diffusion surface area was 0.64 cm^2. The receptor solution was stirred by Teflon coated magnetic stirrers. The donor cell was filled with 0.2 ml of unlabelled NA (15 mg/ml) spiked with 2.5 μCi of hot drug. All permeation experiments were performed at room temperature. Aliquots of 0.2 ml were withdrawn from each receptor compartment over a period of 12 h and replaced with an equal volume of the blank receptor solution to maintain a constant volume. The major factor for setting the duration of permeation studies at 12 h was maintenance of skin viability. The samples were mixed with 10 ml of scintillation cocktail fluid (Packard, Meredien, CT, USA) and the disintegrations per minute (dpms) were counted in a liquid scintillation counter (LSC, Model 500, Beckmann, Fuellerton, CA, USA).

Evaluation of Concentration Gradient of Drug in Skin Layers

Skin sections sliced parallel to the surface of the skin were used for localization of the model drug within each skin layer. Following a 12 h permeation study using the radiolabeled drug, the samples were rinsed carefully with distilled water. Skin samples were placed on dry ice until ready for sectioning. Each skin sample was horizontally cut into sections of 20-μm thickness using a Carl Zeiss HM 505 E microtome (Fisher, PA, USA). Section pieces were collected into 4 groups and labeled from the outermost stratum corneum assigned number 1 to the innermost dermis assigned number 4. The radioactivity of each section was analyzed using LSC.

Analytical Methods

The HPLC pump system for analyzing NA consisted of a pump (Waters 510, Milford, MA, USA), a 4.6 × 150 mm

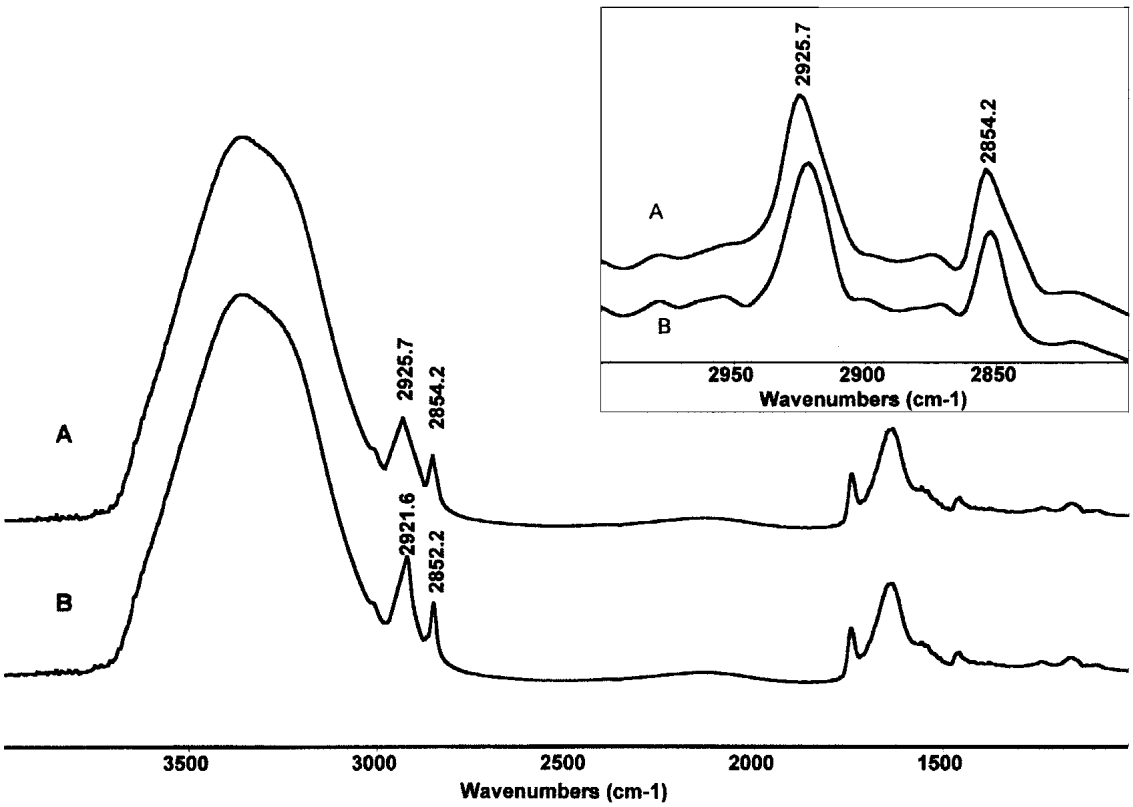

Fig. 2. FTIR spectra of rat dorsal skin (n = 6).

Table I. The Changes in Frequency of Skin Components Using ATR-FTIR

Skin components	A23187 treated	Control
C-H Symmetric Stretching absorbance 2850 cm^{-1}	2854.40 ± 0.40^a	2852.2 ± 0.30^a
C-H Asymmetric Stretching absorbance 2920 cm^{-1}	2925.70 ± 0.60^b	2921.60 ± 0.30^b

[a,b] Significantly different ($p < 0.05$).

Nucleosil C_{18} column, a variable UV detector (Model 486, Waters) and an integrator (Waters). The mobile phase consisted of 2% methanol: potassium phosphate monobasic (pH 7.0). The flow rate of the mobile phase was 1.0 ml/min and the detector wavelength was set at 263 nm.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). The difference in mean flux values determined under the experimental conditions was statistically analyzed by a one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Effects of A23187 on Modulation of the Calcium Gradient in the Skin

A23187 was used to modulate the calcium gradient in the skin. It was shown that A23187 was capable of causing an incubation-time dependent decrease in cell viability (18,19). Skin samples pretreated with A23187 and the control samples were stained with hematoxylin and eosin and observed under a light microscope [Fig. 1, (A)]. Numerous sebaceous glands (S) and hair follicles (F) are embedded in the reticular layer of the dermis. The epidermis is barely seen as a horizontal strip just beneath the stratum corneum (C).

No significant differences were observed in the cellular structures or in the stratum corneum, indicating that pretreatment with A23187 for a period of 1 h did not affect skin viability. Based on this finding, period of 1 h was chosen for the pretreatment of skin samples with A23187.

Since calcium modulation upon exposure to A23187 has been studied using fluorescent calcium probes (20), the immunohistochemistry was used to demonstrate the effect of modulation of calcium gradient in the dermis on the regulation of phosphoproteins, such as Elk-1. Induction of pElk-1 immunoreactivity was examined to see alteration of cellular events by A23187 in both nuclear and cytoplasmic compartments in the dermis. Sections from the controls demonstrated the low level of pElk-1 immunoreactivity throughout the tissue sections except for the sebaceous glands [Fig. 1(B)], while pElk-1 immunoreactivity was significantly enhanced in the nuclear compartments of the A23187 (25 μg/ml) treated samples [Fig. 1(C–F)]. Prominent pElk-1 immunoreactivity was detected in the sebaceous glands [Fig. 1(D)] as well as in the reticular layer [Fig. 1(E, F)] of the dermis as compared to the controls. Moderate pElk-1 immunoreactivity was also detected in the epidermis containing stratum granulosum [Fig. 1(B, C)].

The finding that the pElk-1 immunoreactivity increased in the nuclear and cytoplasmic compartments of the samples suggests that modulation of calcium gradient in the dermis upregulates Elk-1 phosphorylation. Therefore, the alteration of pElk-1 immunorectivity may be a useful *in vivo* marker for the permeation study of a model drug.

ATR-FTIR Spectroscopic Study

ATR-FTIR spectroscopy was used to study the biophysical properties of the skin membrane, including the lipid conformations. The deconvoluted ATR-FTIR spectra for the epidermis-exposed control (a lower curve) and the epidermis-exposed sample treated with A23187 are shown in Fig. 2. Treatment of the rat dorsal skin for 1 h with A23187 resulted in a shift to higher frequency and an absorbance broadening for the C-H symmetric and asymmetric stretching absorbance peaks. The asymmetric stretch peak shifted from 2921.6 ± 0.3

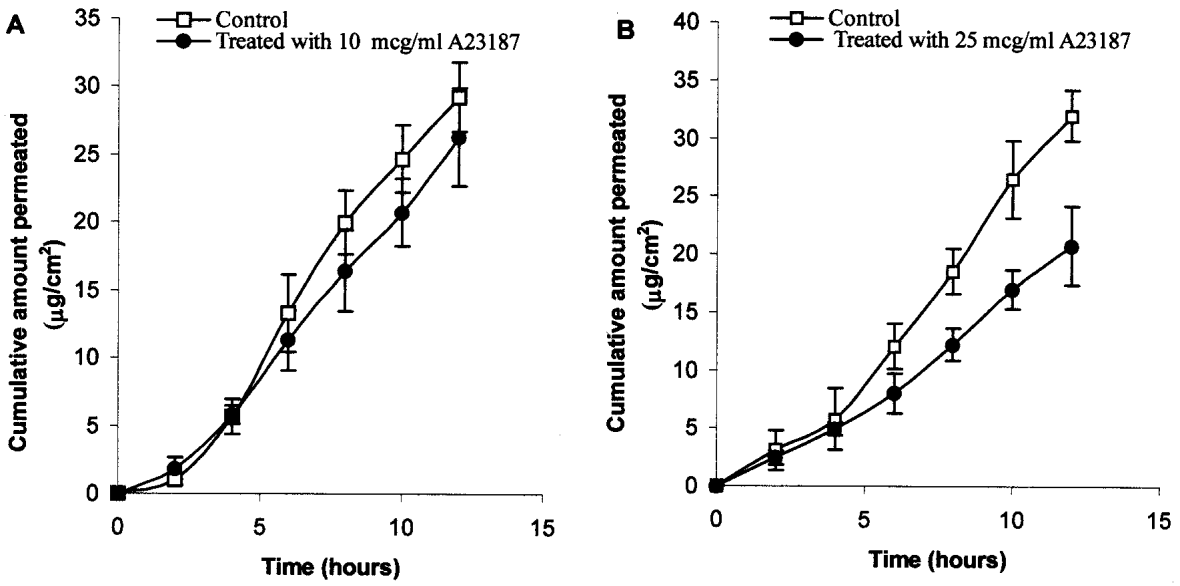

Fig. 3. Permeation profile of nicotinic acid through rat dorsal skin (n = 9).

Table II. The Permeation Rates of Nicotinic Acid through Rat Abdominal Skin Pretreated with A23187 or the Control Samples

Concentration of A23187 (μg/ml)	Skin sample	Flux values (μg/cm²/hr) ± SD
10	Treated	2.9438 ± 0.7470
	Control	3.2455 ± 0.1606
25	Treated	2.2714 ± 0.6040[a]
	Control	3.1895 ± 0.2456[a]

[a] Significantly different ($p < 0.05$).

cm^{-1} (the control) to 2925.7 ± 0.6 cm^{-1} (the sample treated with A23187) (Table I), indicating that treatment of the skin with A23187 leads to a modulation in the calcium gradient and causes a change in the lipid conformation in epidermis and dermis.

A23187 is known for accelerating mucin secretion, which seemed to be accompanied by the lipid conformation change. Because it was proposed that micromolar concentrations of A23187 are capable of stripping phospholipids of divalent cations, such as calcium, the release of bound calcium from calcium binding sites affected the conformation of the lipid bilayer by changing membrane transition temperature or local charged density at the lipid aqueous interface (21). This result, in part, explains the mechanism involved in regulation of the permeation rate of a model drug through the skin, whose calcium gradient was modulated by the cation ionophore.

NA Permeation Study through Rat Dorsal Skin

Because one of the major characteristics of pathologic skin disorder including psoriasis and acne is an abnormal distribution of calcium, and there is a close relationship between the barrier function and the quantity of calcium in the epidermis, the homeostasis of calcium in the skin should be taken into consideration while delivering exogenous compounds through the skin. For this study, NA was selected as a model drug considering its high ionization rate at pH 7.4 and widespread use in percutaneous absorption studies in both animals and humans.

The cumulative amount of NA transported through the rat dorsal skin treated with A23187 increased as a function of time. There was a short lag period at the initial stage as shown in Fig. 3. Slow and steady state permeation of NA through the skin over the entire period (12 h) was observed. No significant difference in the permeation rates of NA was observed between skin samples treated with 10 μg/ml A23187 and the control [Fig. 3(A)], whereas a significant difference was observed at a concentration of 25 μg/ml of A23187 [Fig. 3(B)]. This indicates that there is a threshold concentration of A23187, which is capable of modulating the calcium gradient in the skin and further affecting the percutaneous absorption of a model drug. The amount of NA permeated through the A23187-pretreated skin was found to be significantly lower than that of the control as shown in Table II (2.2714 ± 0.6040 vs. 3.1895 ± 0.2456 μg/cm²/h).

Because no depletion of the drug in the donor compartment occurred, this type of permeation can be described as "infinite". A factor that may be important in the slow diffusion of NA through the skin is its ionization. Since NA has a pk_a of 4.84, most of them are in ionized form in the HHBSS vehicle (pH 7.4). The iso-electric point of keratin is about 5 (22). It is positively charged at pH 2.0 and negatively charged at pH 7.4. The ionized form of NA is a co-ion to the negatively charged keratin and it can be displaced from the membrane due to electrostatic repulsion between the diffusing molecules and skin layer components, such as calcium (23,24,25,26). The ionized forms of NA may penetrate through the skin by the hydrophilic pathway that consists of the aqueous micropores located intercellularly between the laminar lipid structures. A23187 affected the conformation of the lipid bilayer, while displacing calcium. The calcium displacement activity might predominate the lipid conformation change in regulating the permeation rate of our model drug. The results obtained from the permeation studies supported that alteration in the calcium gradient

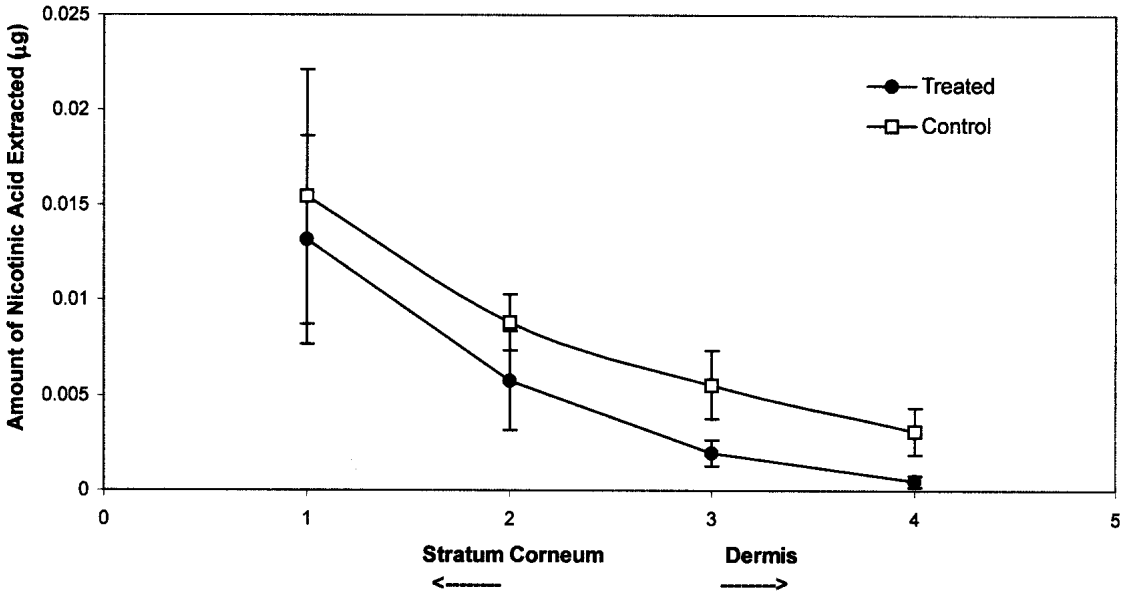

Fig. 4. Distribution profile of nicotinic acid within rat skin 12 h after permeation (n = 9).

significantly affected the permeation profile of a highly ionized drug, nicotinic acid.

Evaluation of Drug Distribution in the Membrane

The results of cryostat sectioning of skin samples, prepared after a 12-h permeation study, demonstrated a concentration gradient of NA with a higher concentration in the stratum corneum and a lower concentration in the dermis. As described in the experimental section, the x-axis in Fig. 4. denotes 4 layers in the skin, assigning the outermost stratum corneum number 1 and the innermost dermis number 4. Between the control and the samples treated with A23187 (25 μg/ml), no significant difference was observed in the amount of NA in the stratum corneum, but a significant difference was observed in the dermis. The results obtained from the cryostat sectioning study indicated that alteration in the calcium gradient significantly affected the drug distribution through the skin layers and the different profiles of the remaining amount of NA in the dermis reflected in the different permeation rates of NA through the skin.

CONCLUSIONS

The effect of an altered calcium gradient in the skin on the percutaneous absorption of a model drug was examined. A change in the calcium gradient may lead to enhance calcium signaling, which may subsequently induce the activation of the phosphoprotein, Elk-1. The change in the calcium gradient caused by A23187 is demonstrated by the immunohistochemistry and the conformational change of lipid bilayers is demonstrated by FTIR study. The change in the calcium gradient further affected the percutaneous absorption of NA, which is ionized at the physiological pH. The concentration gradient of the model drug through the skin measured by the cryostat analysis further support our conclusions.

The results of this study explain the role of calcium in the skin and its effect on the percutaneous absorption of the model drug through the skin. This further suggests that percutaneous absorption of exogenous compounds through the skin can be regulated by calcium. This study may lead to a new strategy for the treatment of skin disorder.

REFERENCES

1. H. Hennings, D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**:245–254 (1980).
2. G. K. Menon, S. Grayson, and P. M. Elias. Ionic calcium reservoirs in mammalian epidermis: Ultra structural localization by ion-capture cytochemistry. *J. Invest. Dermatol* **84**:508–512 (1985).
3. G. K. Menon, P. M. Elias, and K. R. Feingold. Integrity of the permeability barrier is crucial for the maintenance of the epidermal calcium gradient. *Br. J. Dermatol.* **130**:139–147 (1994).
4. G. K. Menon, P. M. Elias, S. H. Lee, and K. R. Feingold. Localization of calcium in murine epidermis following disruption and repair of permeability barrier. *Cell. Tiss. Res.* **270**:503–512 (1992).
5. G. K. Menon and P.M Elias. Ultrastructural localization of calcium in psoratic and normal human epidermis. *Arch. of Dermatol.* **127**:57–63 (1991).
6. E. H. Choi, S. K. Ahn, and S. H. Lee. The changes of stratum corneum interstices and calcium distribution of follicular epithelium of experimentally induced comedones (EIC) by oleic acid. *Exp. Dermatol.* **6**:29–35 (1997).
7. P. W. Reed and H. A. Lardy. A23187: a divalent ion cationic ionophore. *J. Biol. Chem.* **247**:6970–6977 (1972).
8. B. Borle and R. Studer. Effects of calcium ionophores on the transport and distribution of calcium in isolated cells and in liver and kidney slices. *J. Membr. Biol.* **38**:51–72 (1978).
9. T. E. Phillips and J. Wilson. Signal transduction pathways mediating mucin secretion from intestinal goblet cells. *Dig. Dis. Sci.* **38**:1046–1054 (1993).
10. J. Whitmarsh, P. Shore, A. D. Sharrocks, and R. J. Davis. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**:403–407 (1995).
11. G. M. Golden, J. E. Mckie, and R. O. Potts. Role of stratum corneum lipid fluidity in transdermal drug flux. *J. Pharm. Sci.* **76**:25–28 (1987).
12. V. H. W. Mak, R. O. Potts, and R. H. Guy. Oleic acid concentration and effect in human stratum corneum: Non-invasive determination by attenuated total reflectance infrared spectroscopy. *J. Control. Release* **12**:67–75 (1990).
13. Y. Yokomizo and H. Sagitani. Effects of phospholipids on the in vitro percutaneous penetration of prednisolone and analysis of the mechanism by using attenuated total reflectance-Fourier transform infrared spectroscopy. *J. Pharm. Sci* **85**:1220–1226 (1996).
14. L. G. Luna. *Manual of histologic staining methods of the Armed Forces Institute of Pathology,* McGraw-Hill Inc, New York, 1968.
15. S. W. Collier, N. M. Sheikh, J. L. Lichtin, R. F. Stewart, and R. L. Bronaugh. Maintenance of skin viability during in vitro percutaneous absorption and metabolism studies. *Tox. Appl. Pharmacol.* **99**:522–533 (1989).
16. E. S. Choe and J. F. McGinty. Cyclic AMP and mitogen-activated protein kinases are required for glutamate-dependent cyclic AMP response element binding protein and Elk-1 phosphorylation in the dorsal striatum in vivo. *J. Neurochem.* **76**:401–412 (2001).
17. E. S. Choe and J. F. McGinty. N-methyl-D-aspartate receptors and p38 mitogen-activated protein kinase are required for cAMP-dependent cyclase response element binding protein and Elk-1 phosphorylation in the striatum. *Neurosci.* **101**:607–617 (2000).
18. F. A. X. Schanne, A. A. B. Kane, E. E. Young, and J. L. Farber. Calcium dependence of toxic cell death: A final common pathway. *Science* **206**:700–702 (1979).
19. J. Segal and S. H. Ingbar. A postulated mechanism for the coordinate effects of ionophore A23187 on calcium uptake and cell viability in rat thymocytes. *Biochim. Biophys. Acta.* **684**:7–11 (1982).
20. E. N. Dedkova, A. A. Sigova, and V. P. Zinchenko. Mechanism of action of calcium ionophores on intact cells: ionophore-resistant cells. *Membr. Cell Biol.* **13**:357–368 (2000).
21. J. W. Clack, P. S. Brown, J. M. Feldmann, and F. A. Witzmann. A23187 releases bound rather than free calcium from calcium loaded liposomes. *Biochem. Biophys. Res. Commun.* **204**:142–149 (1994).
22. R. C. Marshall. Characterization of the proteins in human hair and nails by electrophoresis. *J. Invest. Dermatol* **80**:519–524 (1983).
23. M. Higa, A. Tanioka, and K. Miyasaka. A study of ion permeation across a charged membrane in multicomponent ion systems as a function of charged membrane density. *J. Membr. Sci.* **49**: 145–169 (1990).
24. T. Kobayashi, T. Nagar, T. Suzuki, Y. Nosaka, and N. Fuiji. Restricted permeation of dextran sulfates by electrostatic barrier of negatively charged ultrafiltration membranes. *J. Membr. Sci.* **86**: 47–56 (1994).
25. T. Hatanaka, M. Inuma, K. Sukibayashi, and Y. Morimoto. Prediction of skin permeability of drugs I. Comparison with artificial membrane. *Chem. Pharm. Bull.* **38**:3452–3459 (1990).
26. T. Hatanaka, K. Katayama, T. Koizumi, K. Sukibayashi, and Y. Morimoto. Time dependent percutaneous absorption enhancing effect of ethanol. *J. Control Release* **33**:423–428 (1992).