Electrophysiological Characterization of Tight Junctional Pathway of Rabbit Cornea Treated with Ophthalmic Ingredients

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The purpose of this study was to investigate the continuous and real-time influence of ophthalmic ingredients on rabbit cornea by monitoring electrophysiological characteristics. The tight junctional permeabilities of FITC-dextran 4400 (FD-4) was also determined through the cornea in the presence of ophthalmic ingredients. Intact cornea showed approximately one k-ohm×cm^2 of transepithelial electrical resistance (TEER) and extremely low permeability of FD-4. The ophthalmic ingredients used in the present study were benzalkonium chloride (BK; 0.002%, 0.01%, 0.05%), ethylenediaminetetraacetic acid (EDTA; 0.5%), capric acid (C10; 0.25%), saponin (SP; 0.1%), taurocholic acid (TA; 1.0%) and sodium dodecyl sulfate (SDS; 0.01%). They were previously reported to be effective on corneal penetrations of various drugs at those concentrations without severe toxicity. These ingredients decreased TEER and increased corneal permeability of FD-4. BK reduced TEER in a concentration-dependent manner. There was a significant correlation (γ=0.860) between the permeability coefficient (Papp) of FD-4 and conductance (Gm), which is the reciprocal value of TEER. It was also indicated that Papp and Gm have a relationship with the corneal cytotoxicity of the ingredients. In conclusion, an electrophysiological method using isolated cornea was very useful to determine the continuous and real-time influence of ophthalmic ingredients on the cornea. In this method, electrophysiological conductance must be able to predict corneal tight junction permeability and the corneal cytotoxicity of ingredients.

Key words eye; cornea; permeability; electrophysiolosy; tight junction

The cornea is generally recognized as the major route of ocular penetration for topically instilled drugs. The corneal composite structures consist of three primary layers, the epithelium, stroma and endothelium. The epithelium, being lipoidal in nature, is considered to contribute to corneal penetration barrier of particularly hydrophilic drugs. An apical tight junction between surface epithelial cells produces the greatest resistance to diffusion across the paracellular pathway. The tight junction primarily restricts the entry of hydrophilic drugs through the paracellular pathway.[1,2)]

The tight junction of the cornea is considered to be influenced by various ophthalmic ingredients. Marsh and Maurice[3)] reported that the concentration of fluorescein in the aqueous humor of humans was enhanced by the instillation of dye with a non-ionic surfactant. Smolen *et al.*[4)] demonstrated that various preservatives or cationic polymers enhanced carbachol permeability and increased drug concentration inside the eye globe. Newton *et al.*[5)] reported that Azone, a transdermal absorption enhancer, increased the ocular delivery of instilled cyclosporine and enhanced immunosuppression activity. We previously reported that ocular absorption of insulin was increased by ethylenediaminetetraacetic acid (EDTA) and benzalkonium chloride (BK),[6)] and corneal penetration of sorbitol was improved by EDTA and chlorhexidine acetate.[7)]

The paracellular pathway can be monitored in real time by continuous measurement of the electrophysiological property of the cornea.[1)] Transepithelial electrical resistance (TEER) is sensitive to changes occurring in paracellular permeabilities of epithelial tissues, and a drop in TEER is often interpreted as indicating an effect exerted by the ingredients.[1)]

To investigate the continuous and real-time influence of ophthalmic ingredients, we designed a Ussing chamber with an adapter to mount rabbit isolated cornea. Using the equipment, the rabbit corneas were monitored for changed electrophysiological characteristics and permeabilities of FITC-dextran 4400 (FD-4) in the presence of ophthalmic ingredients.

MATERIALS AND METHODS

Animals Male Nippon albino rabbits, 2.0—2.5 kg, were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan). The rabbits were starved for 24 h before use but had free access to water. All experiments in the present study conformed to the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23), the ARVO Resolution on the Use of Animals in Research, and the Declaration of Helsinki.

Materials FD-4 (average molecular weight of 4400) was obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Capric acid (C10) was obtained from Katayama Chemical Co., Ltd. (Osaka, Japan). BK, saponin (SP), taurocholic acid (TA) and oxidized glutathione were commercially obtained from Nacalai Tesque, Inc. (Kyoto, Japan). EDTA, sodium dodecyl sulfate (SDS) and all other chemicals were of reagent grade and obtained from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Penetration Experiment Figure 1a shows the Ussing system (World Precision Instruments, FL, U.S.A.) that was used in the penetration experiment. Ag/AgCl half-cells screw into short tubes, which plug firmly into place in the chamber luer ports. Rabbits were sacrificed by injecting an overdose of sodium pentobarbital into a marginal ear vein. The corneal

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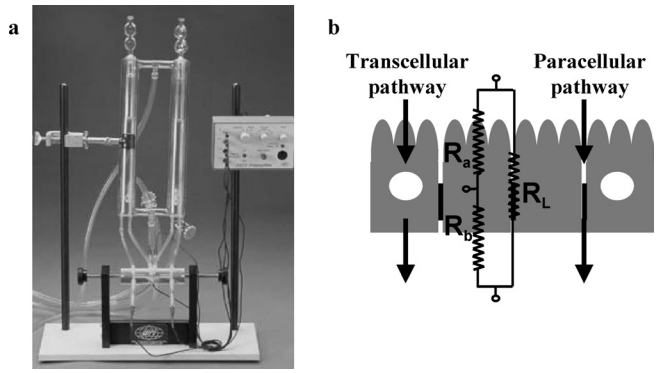

Fig. 1. (a) Ussing System Used in the Penetration Experiment and (b) Equivalent Circuit Model for Corneal Epithelium

membranes were dissected and washed with warm glutathione-bicarbonated Ringer's solution (GBR).[8] GBR contained 106 mM NaCl, 4.8 mM KCl, 0.66 mM $NaH_2PO_4 \cdot 2H_2O$, 29.2 mM $NaHCO_3$, 0.78 mM $CaCl_2 \cdot 2H_2O$, 0.78 mM $MgCl_2 \cdot 6H_2O$, 5.0 mM D-glucose, and 0.15 mM oxidized glutathione. The excised cornea was then immediately mounted on the Ussing chamber CHM 1 (World Precision Instruments) using rubber adapters and O-rings. The chambers were filled with GBR. Aeration and circulation in the tissue bath were provided by means of bubbling with a mixture of 95% O_2 and 5% CO_2. Corneas with high membrane resistance were used in this study. All experiments were conducted at 37 °C using a constant-temperature bath connecting the jacket of the Ussing chambers. A 0.53-cm^2 area of tissue was exposed to the donor and receiver compartments with a volume of 6 ml and 7 ml, respectively.

The electrical output of the Ag/AgCl electrodes was fed to an automatic voltage-clamp unit (CEZ-9100, Nihon Koden, Tokyo, Japan). A spontaneous potential difference (PD) was measured with two matched Ag/AgCl electrodes. Direct current was sent across the tissue with a pair of matched Ag/AgCl electrodes whose tips were positioned away from the tissue surfaces at the far end of two reservoirs. The current flowing in the bath-tissue-bath circuit under short-circuit conditions was monitored. The short-circuit current (Isc) was measured as the current passing through the cornea under zero voltage clamp conditions.

Figure 1b shows an equivalent circuit model for corneal epithelium and electrophysiological parameters (PD, Isc and TEER). PD represents the active ion "pump", presumably located in the basolateral membrane, which maintain the gradients of permeant ions across the cornea.[9] Isc was measured as the current passing through the cornea under zero voltage clamp conditions. In this condition, Isc is indicative of active ion transport[9] since the gradients of permeant ions between the apical membrane and basolateral membrane can be neglected. All of the transepithelial electrical resistance was described as follows: $R = R_L \times (R_a + R_b)/(R_a + R_b + R_L)$. Transepithelial electrical resistance of apical and basolateral cell membranes (R_a and R_b) was much higher than that of tight junctional resistance ($R_a + R_b \gg R_L$), and almost of all the resistance of the corneal membrane showed tight junctional resistance (R_L); therefore, TEER, in general, was sensitive to changes occurring in the paracellular permeabilities of epithelial tissues.[10]

The penetration experiment was started by the addition of penetrant solution (50 μM FD-4) with or without ingredients (0.002% BK, 0.01% BK, 0.05% BK, 0.5% EDTA, 0.25% C10, 0.1% SP, 1.0% TA, and 0.01% SDS) into the donor side after 80 min preincubation, because the electrophysiological parameters reached the steady state within 80 min after mounting the cornea. At 10-min intervals for 160 min, electrophysiological parameters were determined after a sample (500 μl) was withdrawn from the receiver side, and then the same volume of GBR was added to the same side. The apparent permeability coefficient through the overall membrane (Papp, cm/s) was calculated from the slope (flux, nmol/h) of the drug amount *vs.* the time profile on the receiver side (Papp = slope/3600/surface area of the layer/initial concentration on the donor side).

Relative Effect on Cell Viability RCEC were cultured according to an ordinary method reported previously.[9] RCEC were grown using DMEM/F-12 at pH 7.4. The culture medium was supplemented with 5% FBS, 10 ng/ml EGF, 100 ng/ml CTX, 5 μg/ml ISL, 500 ng/ml HCS and the antibiotics (penicillin G 100 IU/ml + streptomycin 100 μg/ml). RCEC were seeded at a density of 2×10^4 cells/cm^2 on a tissue culture plate and cultured at 37 °C under 95% air and 5% CO_2. After 24 h seeding, the culture medium was replaced with fresh medium with or without ingredients. After the addition of 10 μl of WST-1 solution, RCEC were cultured for more than 4 h. Absorbance was measured with a microplate reader (SPECTRA, Tecan, Austria) at 405 nm (reference wavelength 630 nm). The experiments were performed in triplicate. The relative effect on cell viability was calculated as the value of (1-viability)/viability.

Drug Determination The FD-4 sample was determined with a spectrofluorophotometer (FP-770, Jasco, Tokyo, Japan) at an excitation wavelength of 489 nm and emission wavelength of 515 nm.

Data Analysis Papp and the lag time of FD-4 through the corneal membrane were estimated from the slope and X-intercept of the linear portion in plotting the accumulated drug amount in the receiver cell *vs.* time.

Comparisons were made using an unpaired Student's *t*-test for independent samples with Bonferroni correction.

RESULTS AND DISCUSSION

For the corneal absorption of water-soluble drugs and peptides, which are passively absorbed, the paracellular pathway through tight junctions to the lateral intercellular space is an actual transport route. The usefulness of the ocular delivery of cyclosporine, various growth factors, interferons and interleukins has been demonstrated in uveitis, wound healing, herpes simplex, infections and modification of an immune response.[5,11—13] Recently, some ingredients were demonstrated to affect corneal drug permeability. The paracellular pathway of epithelial tissues is often evaluated using an electrophysiological method. Electrophysiological transepithelial resistance is sensitive to changes in the paracellular permeabilities of epithelial tissues.[1]

In the ophthalmic field, it is not easy to conserve the isolated cornea for electrophysiological measurement; therefore, we designed the Ussing chamber with an adapter to mount the rabbit cornea. In the present study, we investigated the

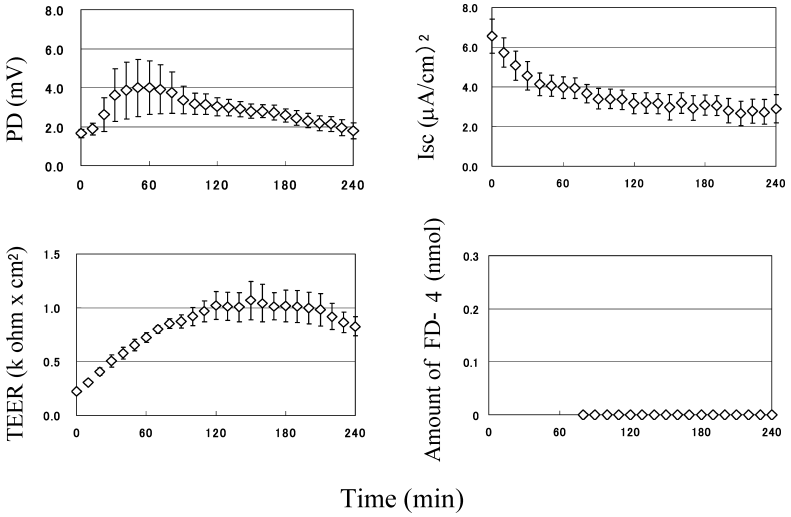

Fig. 2. Electrophysiological Parameters of the Cornea and Penetration Profile of FD-4 through the Cornea

Each value represents the mean±S.E. of at least four experiments.

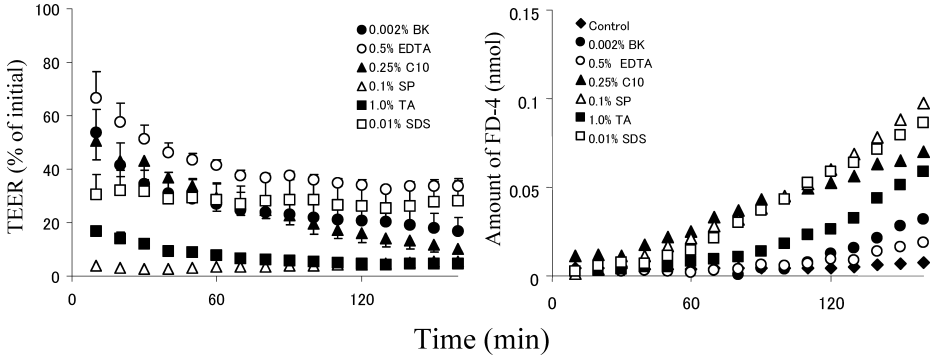

Fig. 3. Effect of Ingredients on Transepithelial Electrical Resistance (TEER) of Cornea

Each value represents the mean±S.E. of at least three experiments. Penetration profile of FD-4 through the cornea treated with various ingredients. Each value represents the mean±S.E. of at least three experiments.

continuous and real-time influence of ophthalmic ingredients on the tight junction of the cornea.

Figure 2 shows electrophysiological parameters of the intact cornea and the penetration profile of FD-4 through the cornea. Corneal Isc and PD are indicative of active ion transport, and corneal TEER is indicative of the changes occurring at the tight junction.[1] These parameters reached the steady state within 80 min after mounting the cornea, as shown in Figure 2. Based on these results, the penetration experiment was started 80 min after mounting the cornea.

Corneal TEER was approximately one k-ohm×cm^2 and FD-4 permeability was extremely low. FD-4, a hydrophilic and high-molecular-weight drug, would permeate through a paracellular pathway by passive diffusion.[14,15] Rojanasakul *et al.*[16] compared the TEER of various epithelial tissues and demonstrated that the corneal membrane is very tight compared with other tissues. In their report, the rank order was as follows: intestinal (211 ohm×cm^2)<nasal (261 ohm×cm^2)<bronchial (266 ohm×cm^2)<tracheal (291 ohm×cm^2)<vaginal (372 ohm×cm^2)<rectal (406 ohm×cm^2)<corneal (1012 ohm×cm^2)<buccal (1803 ohm×cm^2)<skin (9703 ohm×cm^2). Corneal TEER in the present study is consistent with this report. The present and previously reported results indicated that the corneal paracellular pathway is extremely narrow and has a tight junctional barrier for the permeability of hydrophilic macromolecules.

Figure 3 shows the effects of ingredients on TEER of the cornea and the penetration profile of FD-4 through the cornea. TEER profiles were described as percentages of the initial value 80 min after mounting the cornea. The ingredients used included 0.002% BK, 0.5% EDTA, 0.25% C10, 0.1% SP, 1.0% TA, and 0.01% SDS. They were previously reported to be effective for the corneal penetration of various drugs at those concentrations without severe toxicity.[6,7,13,17] The ingredients decreased TEER. The electrophysiological method enables evaluation of the change of the paracellular pathway transiently. The ingredients decreased PD and increased Isc in many cases but the values fluctuated. The decrease of resistance may destroy the ionic balance.

Surfactants such as BK, SDS, and SP were reported to be incorporated into the lipid bilayer at low concentrations, and to change the physical properties of cell membranes.[18] It is conceivable that mixed micelles begin to form, resulting in the removal of phospholipids from cell membranes and also membrane solubilization when the lipid bilayer is saturated. Consequently, these surfactants are considered to regulate the tight junction.[18] In the case of calcium chelators, Ca^{2+} depletion does not act directly on the tight junction, but rather in-

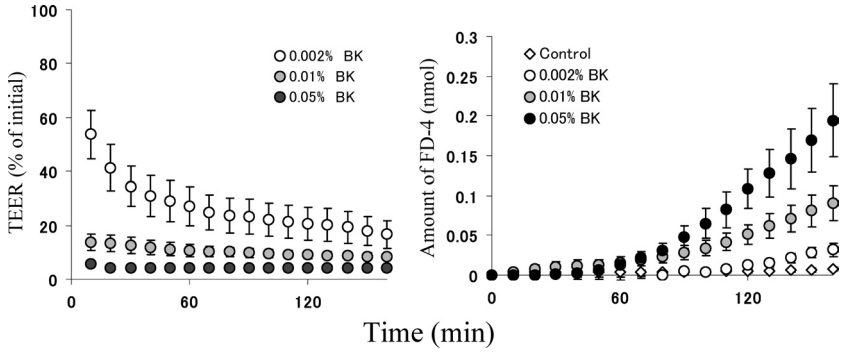

Fig. 4. Effect of BK Concentration on Transepithelial Electrical Resistance (TEER) of the Cornea

Each value represents the mean±S.E. of at least three experiments. Effect of BK concentration on penetration of FD-4 through the cornea. Each value represents the mean±S.E. of at least three experiments.

Table 1. Apparent Permeability Coefficient (Papp), Lag Time of FD-4 Treated with Various Ingredients

Absorption enhancers	*n*	Lag time (min)	Papp ($\times 10^{-6}$ cm/s)	Ratio[a)]
Control	6	99.2	0.044	1.0
0.002% BK	3	97.3	0.313*	6.3
0.01% BK	4	65.5	0.615*	12.4
0.05% BK	4	71.4	1.360*	27.1
0.5% EDTA	4	71.8	0.152	3.1
0.25% C10	5	10.8	0.259*	5.5
0.1% SP	4	43.9	0.573*	11.5
1.0% TA	4	85.8	0.487*	9.8
0.01% SDS	3	16.2	0.360*	7.2

a) Ratio of Papp to control. $*p<0.05$, significantly different from control.

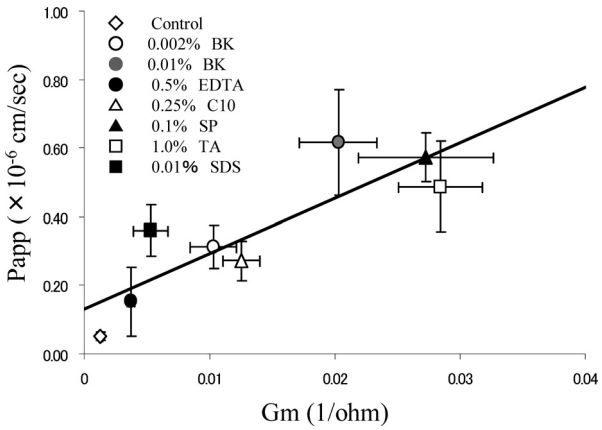

Fig. 5. Relationship between Conductance (Gm) and Apparent Permeability Coefficient (Papp)

Each value represents the mean±S.E. of at least three experiments.

duces global changes in the cells, including the disruption of actin filaments, disruption of adherent junctions, diminished cell adhesion and activations of protein kinases.[19,20)]

Papp and lag time are summarized in Table 1. Ophthalmic ingredients significantly increased the corneal permeability of FD-4 3.1—27.1 times. BK enhanced the corneal penetration of FD-4 in a concentration-dependent manner. It is important to notice that BK influenced the tight junction of the corneal epithelium even at commercially used concentrations (0.002% and 0.01%). The ingredients showed different lag times, suggesting that ingredients affect the stroma and endothelium differently. Further study is necessary to investigate the mechanism of ingredients in the corneal composite structure.

BK is a powerful cationic detergent that destroys bacteria after ionic attraction[16)] and is frequently used in ophthalmic drug preparations at concentrations between 0.002% and 0.01%.[21)] Figure 4 shows the effect of BK concentration on TEER and the penetration of FD-4 through the cornea. BK decreased TEER in a concentration-dependent manner, although TEER of the cornea treated with 0.05% BK was too small to detect under the present conditions after 20 min.

C10 increases intracellular calcium levels through interaction with phospholipase C in the membrane, and enhances permeability by opening the tight junction through the activation of calmodulin-dependent contraction of the actin microfilament by released calcium.[19,20,22—25)]

It was reported that bile salt such as taurocholic acid enhanced permeability by removing epithelial cells, which constitute a major permeability barrier.[26,27)] For bile salts, the rank order of the promoting effect was sodium deoxycholate>sodium glycocholate>sodium taurocholate.[28)] In addition, Gordon *et al.*[29)] suggested that bile salts might interact with cell membranes to form a reverse micelle, which acts as a channel increasing the permeability of test compounds.

Figure 5 shows the relationship between conductance (Gm) at the end of penetration experiments and Papp. Gm is the reciprocal value of TEER. Overall, there was a significant correlation between Papp of FD-4 and Gm ($\gamma=0.860$); however, there was a slight difference of Gm from the according Papp. Most ingredients affect not only tight junctions but also cell membranes.[18)] Namely, the functional change of the cell membrane by the present ingredients may cause a decrease of corneal TEER or increase the transcellular permeability of FD-4.

The relative effect of ingredients on cell viability of RCEC was assessed using the WST-1 assay, which measures mitochondrial dehydrogenase activity and is used as a measure of toxicity since mitochondrial dehydrogenases are active in living but not in dead cells. The relative effect on cell viability was calculated as the value of (1-viability)/viability. As shown in Figure 6, there was a significant correlation between the relative effect on cell viability and Papp of FD-4 ($\gamma=0.898$), and between the relative effect on cell viability and Gm ($\gamma=0.891$). These results indicated that most ingre-

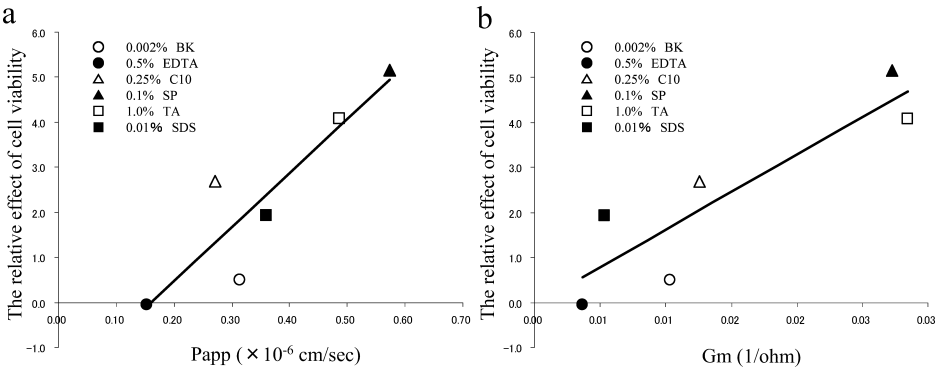

Fig. 6. Relationship (a) between the Relative Effect on Cell Viability and Apparent Permeability Coefficient (Papp) and (b) between the Relative Effect on Cell Viability and Conductance (Gm)

dients might influence the corneal tight junction by a mechanism similar to the relative effect on cell viability.

In conclusion, an electrophysiological method using isolated cornea was very useful to determine the continuous and real-time influence of ophthalmic ingredients on the cornea. In this method, electrophysiological conductance must be able to predict the corneal tight junction permeability of ophthalmic ingredients and their influence on corneal viability.

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