Regular Article

Characterization of Ocular Iontophoretic Drug Transport of Ionic and Non-ionic Compounds in Isolated Rabbit Cornea and Conjunctiva

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Ocular iontophoresis (IP) in isolated rabbit cornea and conjunctiva was examined in terms of transport enhancement, tissue viability and integrity using electrophysiological parameters by the Ussing-type chamber technique. Lidocaine hydrochloride (LC, a cationic compound), sodium benzoate (BA, anionic compound), and fluorescein isothiocyanate labeled dextran (molecular weight 4400 Da, FD-4, hydrophilic large compound) were used as model permeants. Direct electric current was applied at 0.5–5.0 mA/cm^2 for the cornea and 0.5–20 mA/cm^2 for the conjunctiva for 30 min. LC and BA fluxes across the cornea and conjunctiva were significantly increased by the application of electric current up to 2.3- and 2.5-fold and 4.0- and 3.4-fold, respectively, and returned to their baseline level on stopping the current. Furthermore, a much higher increase by IP application was obtained for the FD-4 transport. The increased FD-4 flux in the conjunctiva returned to baseline on stopping the current, whereas the flux in the cornea was sustained at a higher level after stopping the current. The transepithelial electric resistance of the cornea and conjunctiva was lowered by electric current application but fully recovered after stopping the current up to 2.0 mA/cm^2 for the cornea and 10 mA/cm^2 for the conjunctiva, suggesting that the corneal and conjunctival viability and integrity are maintained even after application of these current densities. These results indicate that ocular IP may be a useful non-invasive technique to achieve drug delivery of hydrophilic large molecules into the eyes.

Key words ocular drug delivery; physical enhancement; iontophoresis; electric current; cornea; conjunctiva

Eye drops are used for treatment of ocular diseases and the drugs applied by the eye drops distribute onto the ocular surface, then penetrate into the eye through the ocular surface tissues such as cornea and conjunctiva.[1)] In spite of the simple and convenient dosage form of eye drops, elimination and dilution of drugs by turn-over of tear fluid and the low permeability of the surface ocular tissues are major challenges that limit the absorption of an effective amount of drugs into the eyes.[2)] Thus, approaches to increase drug absorption into the eyes have attracted much attention. One includes chemical approaches such as the use of prodrugs, liposomes, cyclodextrins, and chemical enhancers, while others include physical approaches such as iontophoresis (IP) and sonophoresis.[3,4)]

IP is known as an useful technique to increase the penetration of ionic and non-ionic compounds into the body across surface epithelial tissues including skin and the ocular surface.[5,6)] This technique has a potential to regulate the drug delivery depending upon the applied current density and application time and, thus, provides an ON/OFF control. A number of *in vivo* ocular IP studies have shown that application of IP increases the drug concentration in the aqueous and vitreous humors compared to instillation with gentamicin,[7–9)] vancomycin,[10)] tobramycin,[11,12)] carboplatin,[13)] methylprednisolone[14)] and dexamethasone.[15)] In addition, corneal and conjunctival IP are expected to deliver large molecules having high pharmacological activities with small amount such as antisense oligonucleotides and antibody fragments of growth factors for treatment of corneal angiogenesis, glaucoma, macular degeneration and retinopathy.[16,17)] *In vitro* studies in isolated corneal and conjunctival/scleral tissues have demonstrated that the application of IP increases the permeability of therapeutic compounds such as betaxolol,[18)] timolol,[18,19)] dexamethasone,[19)] methylprednisolone,[20)] and three single stranded oligonucleotide,[21)] depending on the applied current density and duration. Thus, corneal and conjunctival/scleral IP are promising approaches for the intraocular delivery of therapeutic drugs. However, there remains the question of the effect of the electric current on the viable ocular tissues. Thus, the potential effect of the electric current on both transport enhancement and viability of the tissues were characterized in order to establish the usefulness of ocular IP as an enhancing technology. Electrophysiological parameters such as potential difference (PD), short-circuit current (I_{sc}) and transepithelial electrical resistance (TEER) are generally used for monitoring the functions of epithelial tissues,[22–24)] and cell layers.[25)] These parameters have been used as indices of viability and integrity of the tissues for characterization of transport enhancement by chemical enhancers in the isolated cornea and conjunctiva.[26,27)]

In our previous pilot study, the IP effect using an applied current up to 0.85 mA/cm^2 was examined in the isolated rabbit conjunctiva.[28)] The conjunctival transport flux of ionic and non-ionic compounds showed an increase from the baseline flux and a return to baseline when the current application was turned on and off, respectively. The electrophysiological parameters of the isolated conjunctiva were influenced by the current application, then fully returned to baseline after the current application was stopped. Although the ocular IP was partly characterized in the conjunctiva, systematic studies in-

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cluding the potential relationship between transport enhancement and tissue properties has not been conducted.

The purpose of the present study was to characterize the permeation enhancement of ionic and non-ionic compounds in the ocular IP using the isolated cornea and conjunctiva. Lidocaine hydrochloride and sodium benzoate were used as ionic compounds and FD-4 as a non-ionic and hydrophilic large molecule to estimate IP effect for large molecules such as antisense oligonucleotides and antibody fragments. The electrophysiological parameters of the cornea and conjunctiva before and after IP application were measured to monitor the viability and integrity of the tissues. We attempted to understand the potential relationship between the increase in permeation and state of the ocular tissues under the influence of an electric current.

MATERIALS AND METHODS

Chemicals Lidocaine hydrochloride monohydrate (molecular weight (MW): 288.81, LC), sodium benzoate (MW: 144.11, BA) and fluorescein isothiocyanate (FITC)-dextran (MW: 4400, FD-4) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). 2-Morpholinoethanesulphonic acid monohydrate (MES) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and methanol and acetonitrile HPLC grade for HPLC assay were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals were of analytical grade and used without further purification.

Animals Male Japanese white rabbits, weighing 2.5 to 3.5 kg, were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). All animal experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of Josai University.

Buffer Solution Unless otherwise indicated, all experiments were carried out in bicarbonated Ringer's solution (BRS) maintained at 37°C and pH 7.4 under 95% O_2/5% CO_2. BRS was composed of 111.5 mM NaCl, 4.8 mM KCl, 29.2 mM $NaHCO_3$, 0.75 mM $NaHPO_4$, 1.04 mM $CaCl_2$, 0.74 mM $MgCl_2$, and 5 mM D-glucose. The osmolality of the solution was approximately 280 mOsm/kg H_2O and BRS containing 15 mM MES (MES-BRS) at pH 5.9 was used as a buffer for LC to maintain it in an ionic form.

Preparation of Cornea and Conjunctiva The rabbits were anesthetized with an injection of 20 mg/kg sodium pentobarbital solution into a marginal ear vein, and sacrificed by injection with 3.3 M KCl solution into the same ear site. The entire eyeball was then removed from the orbit carefully, the cornea and conjunctiva were isolated and trimmed, and then mounted carefully onto a tissue adaptor with an effective circular area of 0.44 cm^2. The tissue adaptor was then placed between Ussing-type chambers maintained at 37°C using a water jacket.

Measurement of Electrophysiological Parameters Unless otherwise indicated, all experiments were performed under open-circuit conditions with intermittent application of an electric current by a voltage clamp unit (CEZ-9100, Nihon Kohden, Tokyo, Japan). The PD was measured with two matched calomel electrodes. Two salt-agar bridges (containing 4% agar in 3.3 M KCl), the tips of which were located near the center of the tissue surfaces, were used to electrically connect the reservoir fluids to the electrode wells. The electrical output of the calomel electrodes was amplified by a voltage-clamp unit. Direct current across the tissue was transmitted *via* a pair of matched Ag/AgCl electrodes with conducting agar bridges, the tips of which were positioned away from the tissue surfaces at the far ends of the reservoirs. The I_{sc} flowing in the bath-tissue-bath current was monitored intermittently. TEER was calculated by Ohm's law to plug the variation of I_{sc} at 5 and 3 mV pulses to the cornea and conjunctiva, respectively. TEER during IP application was calculated by Ohm's law to plug in PD during the application of a constant electric current density. At a PD over 200 mV during electrical current application, a digital multimeter (PC700, Sanwa Electric Instrument Co., Ltd., Tokyo, Japan) was used instead of CEZ-9100. In the case of the current application with the PC700, the PD of the tissues was compensated for by subtracting the blank PD value without tissue from the value with tissue. TEER during the electric current application was compensated for with a similar calculation to that described above.

Application of Electric Current Electric currents for IP studies ranged from 0.5–5.0 for the cornea and 0.5–20 mA/cm^2 for the conjunctiva. The electric current applications were conducted using the CEZ-9100 for a lower current range of 0.5–2.0 mA/cm^2 and an electric stimulator (SEN-8203, Nihon Kohden, Tokyo, Japan) with an isolator (SS-104J, Nihon Kohden) for a higher current range of 5.0–20 mA/cm^2.

IP Permeation Study In the study with cornea, the chamber facing the epithelial side (donor side) was filled with 3 mL BRS, whereas the chamber facing the endothelial side (receptor side) contained 7 mL BRS. The volume difference between the two chambers was used to maintain the corneal configuration. In the study with conjunctiva, the chambers of both the mucosal (donor) and serosal (receptor) sides were filled with 5 mL BRS. After the PD, I_{sc} and TEER of the tissues were equilibrated, and permeation experiments were initiated by adding the drugs to the donor chamber at 0.5 mg/mL for LC, 2.0 mg/mL for BA, and 2.5 (cornea) or 5.0 (conjunctiva) mg/mL for FD-4. Then, 120 min after the initiation of the permeation experiments, a constant electric current were applied for 30 min as described above. Anodal IP, in which the anode was placed in the donor chamber, was used in the experiments with LC and FD-4, whereas cathodal IP where the cathode was in the donor chamber was used for BA. An adequate amount of sample solution was collected from the receptor chamber at predetermined times, and PD, I_{sc} and TEER were monitored during the IP experiments.

Measurement of FD-4 Retention and Release from Tissues The experimental conditions were the same as those described above. At 120 min after addition of FD-4, a constant current at 0.5 or 5.0 mA/cm^2 was applied for 30 min in the corneal study, while 2.0 or 10 mA/cm^2 was applied for 30 min in the conjunctival study. Both donor and receptor solutions were replaced by superfusion with 100 mL fresh BRS at the end of the current application. The cornea and conjunctiva were removed from the Ussing chamber setup at the end of the current application and at 210 min (cornea) or 150 min (conjunctiva) after replacement of the chamber solution, then rinsed with fresh BRS, and the edge around the effective permeation area was carefully excised. The tissue surface was wiped with KimWipes (Nippon Paper Crecia Co., Ltd., Tokyo, Japan), then the tissues were weighed and shredded into the small pieces with scissors. The resulting pieces were homog-

enized in BRS for 5 min at 4°C, then centrifuged at 10000×***g*** for 5 min at 4°C. The resulting supernatant was subjected to FD-4 assay.

FD-4 release from the cornea and conjunctiva was measured after replacement of the chamber solutions by superfusion at the end of the current application. Aliquots were collected from the receptor chamber at predetermined times, then the sample solution was subjected to FD-4 assay.

Analytical Methods LC and BA in the collected samples were measured by an HPLC system (system controller: SCL-10A_{VP} (Shimadzu, Kyoto, Japan), auto injector: SIL-10A_{XL} (Shimadzu), pump: LC-10A_{VP} (Shimadzu), degasser: DGU-12A (Shimadzu), octadecyl reversed-phase column: Mightysil RP-18 GP (250 mm×4.6 mm, 5 μm, Kanto Chemical Co., Inc.), column oven: CTO-10A (Shimadzu)) connected to a UV detector (SPD-10A_{VP}, Shimadzu). The quantitative measurement of LC was performed as follows: mobile phase: 5 mM KH_2PO_4 solution (pH 2.0)–CH_3OH–acetonitrile 70 : 15 : 15 (v/v/v), flow rate: 1.0 mL/min, column oven: 40°C, detection wavelength: 262 nm, injection volume: 70 μL, internal standard: 1.0 μg/mL *p*-hydroxybenzoic acid. The quantitative measurement of BA was performed as follows: mobile phase: 5 mM KH_2PO_4 solution (pH 2.2)–acetonitrile 50 : 50 (v/v), flow rate: 1.0 mL/min, column oven: 40°C, detection wavelength: 230 nm, injection volume: 20 μL. An absolute calibration method was used.

The fluorescence of FD-4 in the collected samples was measured using a multi-label reader (ARVO™-X3 system, PerkinElmer, Inc. Japan Co., Ltd.) at an excitation and emission wavelength of 485 and 535 nm, respectively.

Data Analysis Unidirectional fluxes (J, μg/cm^2/min) for LC, BA and FD-4 in the permeation study were estimated from the slope of a plot of the cumulated penetrant appearing in the receiver fluid *versus* time. The released flux of FD-4 from ocular tissues was also estimated from the slope of the release profiles. All data were presented as the mean±standard error (S.E.) of 3–8 experiments. Statistical significance among group means was determined by Student's *t*-test. For the amount of FD-4, statistical significance among group means was determined by the Tukey–Kramer test. A $p<0.05$ was considered as statistically significant.

RESULTS

Effect of Electric Current on the Permeation of LC, BA and FD-4 in the Cornea and Conjunctiva In the present study, LC and BA were used as cationic and anionic model permeants, respectively, and FD-4 was a large hydrophilic compound. Figures 1 and 2 show the time–courses of the permeation fluxes of LC and BA, respectively, across the cornea (panel A) and conjunctiva (panel B) in the iontophoretic experiments. Tables 1A and B summarize the baseline and maximal fluxes, and enhancing ratios calculated from the fluxes in the cornea and conjunctiva, respectively. The IP was applied for 30 min from 120 min after beginning the permeation study and, thus, the baseline flux before 120 min served as a control. The apparent permeability coefficient (P_{app}) calculated from the baseline flux of LC across the cornea and conjunctiva was 3.98±0.25 and 3.58±0.19 ($\times10^{-5}$ cm/s), respectively, and 0.83±0.05 and 1.56±0.07 ($\times10^{-6}$ cm/s) for BA across the cornea and conjunctiva. The baseline P_{app} of FD-4 across the conjunctiva was 1.14±0.13 ($\times10^{-7}$ cm/s), whereas the corneal P_{app} of FD-4 was not calculated because the permeation was low. The baseline P_{app} of LC, BA and FD-4 used as model permeants in present study was close to the values reported in previous studies, and the IP permeation experiment considered as suitable.[29–32] Both LC and BA fluxes across the cornea and conjunctiva were increased by the current application and returned to baseline after stopping the current application. LC and BA fluxes in the cornea were increased 2.27- and 2.45-fold compared with baseline by a current application up to 5.0

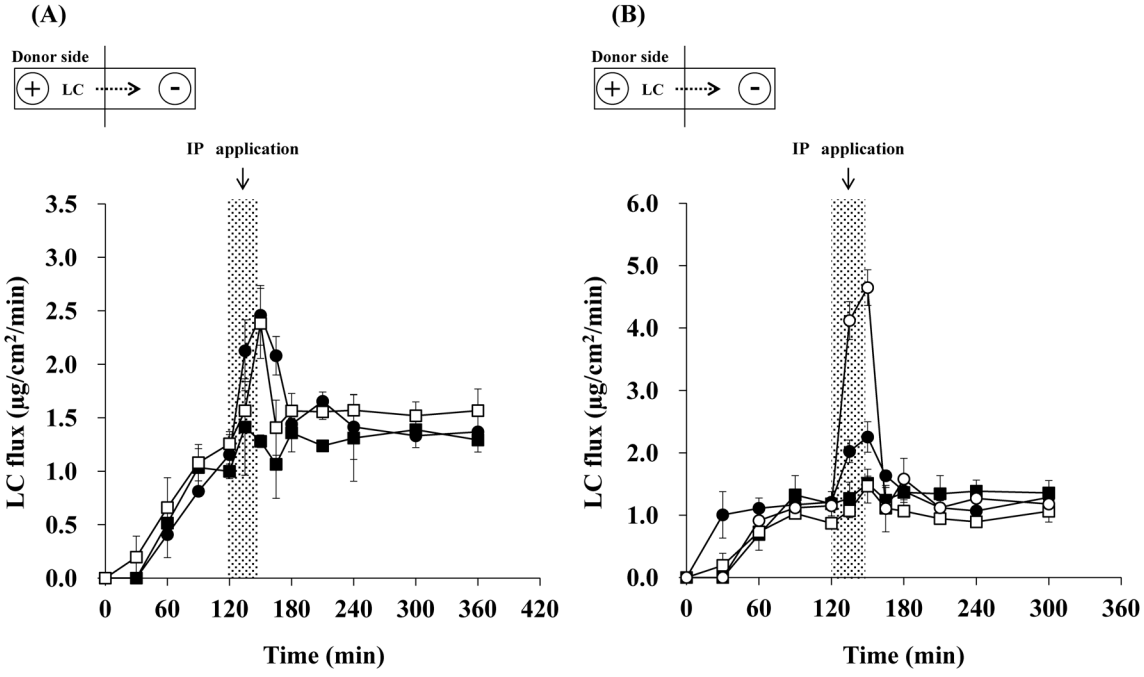

Fig. 1. Time–Courses of Permeated Flux of LC through the Cornea (A) and Conjunctiva (B) in the Anodal IP Experiments

■, 0.5 mA/cm^2; □, 2.0 mA/cm^2; ●, 5.0 mA/cm^2; ○, 10 mA/cm^2. Data represent the mean±S.E. (n=3–5).

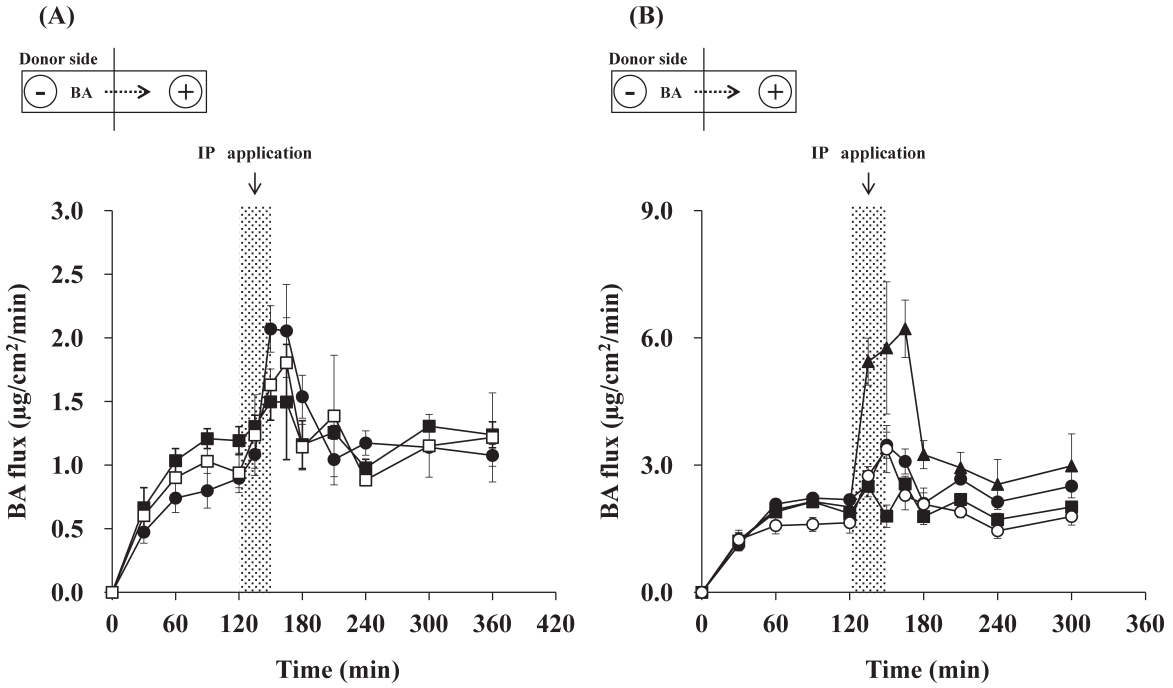

Fig. 2. Time–Courses of Permeated Flux of BA through the Cornea (A) and Conjunctiva (B) in the Cathodal IP Experiments

■, 0.5 mA/cm^2; □, 1.5 mA/cm^2; ●, 2.0 mA/cm^2; ○, 5.0 mA/cm^2; ▲, 7.5 mA/cm^2. Data represent the mean±S.E. (n=3).

Table 1A. Enhancement Ratio and Flux Values of LC, BA and FD-4 in the Cornea as Measured by IP Experiments

Compound	Current density (mA/cm^2)	Baseline flux (μg/cm^2/min)	Maximal flux (μg/cm^2/min)	Enhancement ratio
LC				
	0.5	1.00±0.07	1.65±0.32	1.65
	1.0	1.27±0.20	1.96±0.30**	1.54
	2.0	1.26±0.12	2.41±0.32*	1.92
	5.0	1.16±0.19	2.62±0.24*	2.27
BA				
	0.5	1.19±0.11	1.81±0.21*	1.52
	1.0	0.93±0.07	1.73±0.21*	1.85
	1.5	0.94±0.16	1.95±0.29*	2.08
	2.0	0.90±0.07	2.20±0.30*	2.45
FD-4				
	0.5	N.D.	0.01±0	Acalculia
	1.0	N.D.	0.03±0	Acalculia
	2.0	N.D.	0.05±0.01	Acalculia
	5.0	N.D.	0.06±0	Acalculia

*p<0.05, **p<0.01 *vs.* significantly different from baseline flux (t-test). N.D. not detected. Data represent the mean±S.E. (n=3–5).

Table 1B. Enhancement Ratio and Flux Values of LC, BA and FD-4 in the Conjunctiva as Measured by IP Experiments

Compound	Current density (mA/cm^2)	Baseline flux (μg/cm^2/min)	Maximal flux (μg/cm^2/min)	Enhancement ratio
LC				
	0.5	1.18±0.20	1.57±0.17**	1.33
	1.0	0.95±0.15	1.40±0.22*	1.47
	2.0	0.87±0.02	1.49±0.25	1.71
	5.0	1.21±0.04	2.41±0.18**	1.99
	10	1.15±0.07	4.65±0.29**	4.04
BA				
	0.5	1.86±0.10	2.59±0.21	1.39
	1.0	1.65±0.26	2.21±0.31*	1.34
	2.0	2.19±0.10	3.70±0.19**	1.69
	5.0	1.64±0.24	3.54±0.42**	2.16
	7.5	2.01±0.13	6.92±0.89*	3.44
FD-4				
	0.5	0.03±0.01	0.10±0.01***	3.42
	1.0	0.04±0.01	0.16±0.05	3.58
	2.0	0.05±0.01	0.45±0.04**	8.93
	5.0	0.03±0.01	0.32±0.03***	10.66
	10	0.04±0.01	1.15±0.13*	28.46
	20	0.02±0	1.66±0.28*	83.01

*p<0.05, **p<0.01, ***p<0.001 *vs.* significantly different from baseline flux (t-test). Data represent the mean±S.E. (n=3–8).

and 2.0 mA/cm^2 in a current-dependent manner, respectively (Table 1A). In contrast, LC and BA fluxes in the conjunctiva were increased 4.04- and 3.44-fold compared with baseline by a current application up to 10 and 7.5 mA/cm^2 in a current-dependent manner, respectively (Table 1B). As shown in Fig. 3, a good correlation between PD during the current application and the maximal flux was observed for the cornea (Fig. 3A, LC: r=0.8922, BA: r=0.8808) and the conjunctiva (Fig. 3B, LC: r=0.9582, BA: r=0.8930).

Figure 4 shows the results of FD-4. The conjunctival FD-4 fluxes were increased up to 83.01-fold higher than the baseline flux by the current application in a current-dependent manner (Fig. 4B, Table 1B), then returned to baseline after stopping the current, although the flux after application of 20 mA/cm^2 did not recover sufficiently. The corneal FD-4 fluxes were detected by the current application and the increased fluxes were maintained until termination of the permeation experiments after stopping the current depending on the applied current

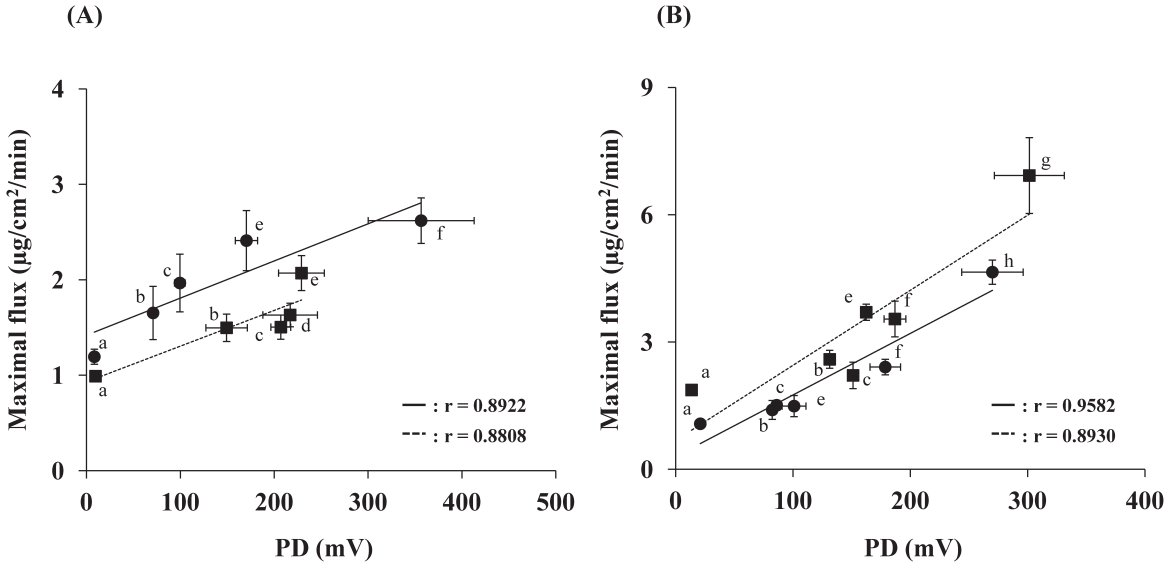

Fig. 3. Relationship between the Corneal (A) and Conjunctival (B) PD and Maximal Permeated Fluxes of LC (●) and BA (■)

a, baseline; b, 0.5 mA/cm^2; c, 1.0 mA/cm^2; d, 1.5 mA/cm^2; e, 2.0 mA/cm^2; f, 5.0 mA/cm^2; g, 7.5 mA/cm^2; h, 10 mA/cm^2. Solid line is the regression line for LC and dotted line for BA. Data represent the mean±S.E. (n=3–5).

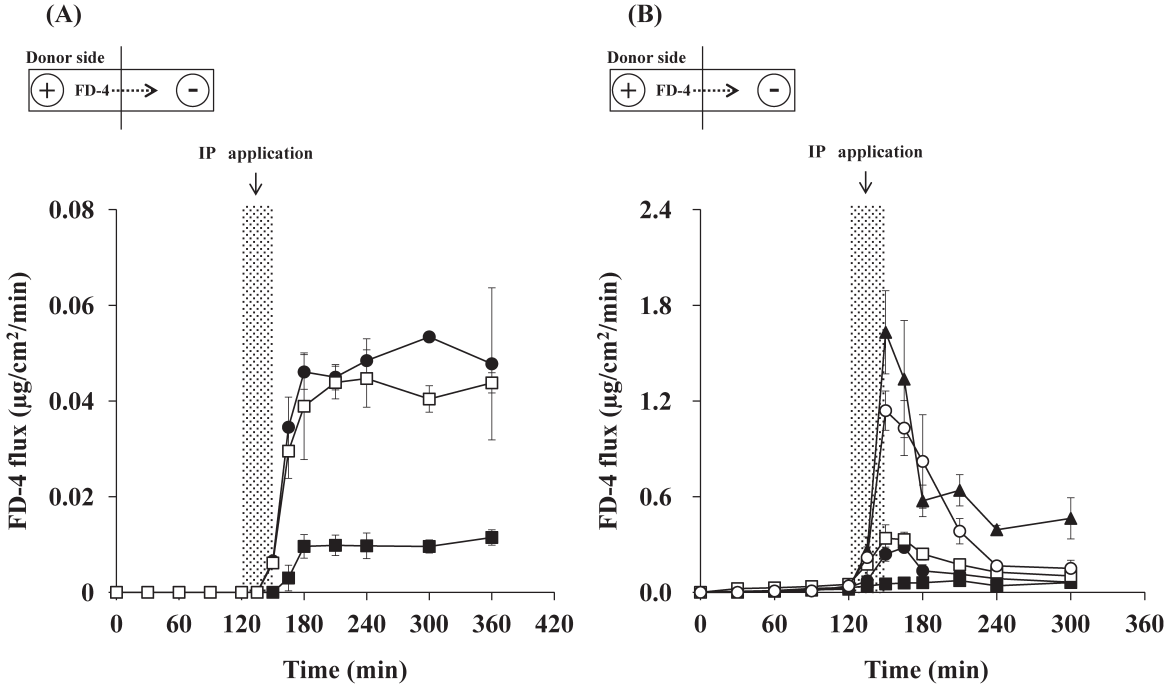

Fig. 4. Time–Courses of Permeated Flux of FD-4 through the Cornea (A) and Conjunctiva (B) in the Anodal IP Experiments

■, 0.5 mA/cm^2; □, 2.0 mA/cm^2; ●, 5.0 mA/cm^2; ○, 10 mA/cm^2; ▲, 20 mA/cm^2. Data represent the mean±S.E. (n=3–8).

up to 5.0 mA/cm^2 (Fig. 4A, Table 1A). The maximal fluxes in conjunctiva achieved by the current application were proportional to PD up to *ca.* 700 mV (Fig. 5B), whereas the corneal fluxes were proportional up to *ca.* 150 mV and show the plateau at higher PD (Fig. 5A). Thus, the enhancing properties of ocular IP for large hydrophilic permeants in the cornea and conjunctiva seem to be different. These results show that higher enhancing effect following IP application can be obtained with large hydrophilic molecules such as FD-4 in the cornea and conjunctiva compared with LC and BA.

FD-4 Retention in the Cornea and Conjunctiva after IP Figures 6A and B show the amount of FD-4 in the cornea and conjunctiva, respectively, immediately after current application and at the end of the IP experiments (at 210 min for the cornea and 150 min for the conjunctiva) after stopping the current. The amount of FD-4 in the cornea and conjunctiva was significantly increased depending upon the applied current. In the conjunctiva, the amount of FD-4 at 150 min after termination of the IP application was reduced to the control level (Fig. 6B, hatched column). The release flux of FD-4 from the conjunctiva after IP application (Fig. 7B) showed an initial increase, followed by a time-dependent decrease similar to the flux profile obtained after IP application in the normal IP permeation experiments (Fig. 4B). In the cornea, the amount of

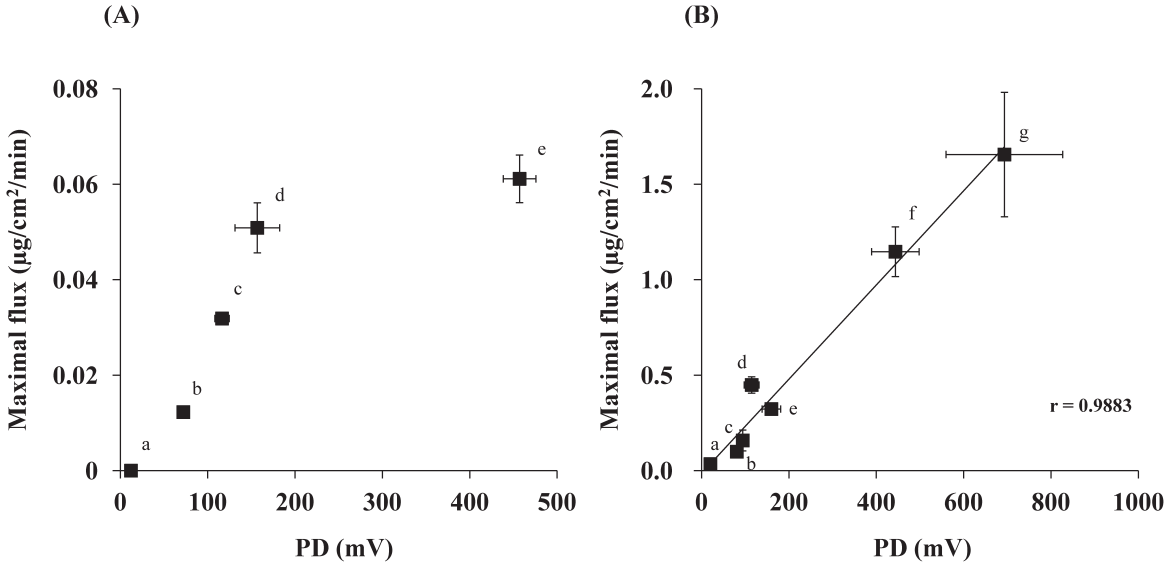

Fig. 5. Relationship between the Corneal (A) and Conjunctival (B) PD and Maximal Permeated Fluxes of FD-4

a, baseline; b, 0.5 mA/cm^2; c, 1.0 mA/cm^2; d, 2.0 mA/cm^2; e, 5.0 mA/cm^2; f, 10 mA/cm^2; g, 20 mA/cm^2. Data represent the mean±S.E. (n=3–8).

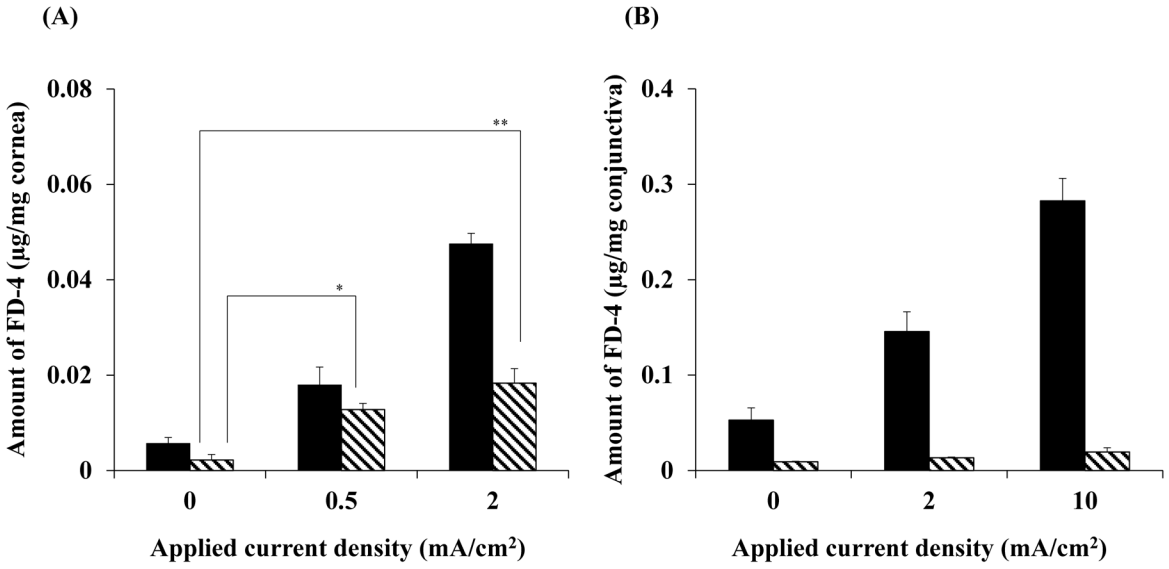

Fig. 6. Retained Amount of FD-4 in the Cornea (A) and Conjunctiva (B) after Anodal IP Application

Closed bars are the FD-4 concentration immediately after IP application. Hatched bars are the FD-4 concentration at 210 min for the cornea and 150 min for the conjunctiva after IP application. *p<0.05, **p<0.01 *vs.* significantly different from 0 mA/cm^2 (Tukey–Kramer test). Data represent the mean±S.E. (n=3–4).

FD-4 at 210 min after IP application was also reduced, but the reduction was significantly higher than for the control (Fig. 6A). In accordance with the amount of FD-4, the release flux from the cornea at 210 min after IP application was markedly obviously higher than the control (Fig. 7A). Thus, the difference in FD-4 flux profiles after IP application between cornea and conjunctiva shown in Figs. 4A and B may be attributed to retention by these tissues.

Effect of Current Application on the Electrophysiological Parameters Electrophysiological parameters were monitored over the period of IP permeation study, thus, the baseline values before IP application served as a control. Figures 8 and 9 show the time courses of the TEER in the cornea (panel A) and conjunctiva (panel B) obtained in the IP experiments. In both the cornea and conjunctiva, the TEER values were drastically reduced by the applied current regardless of the direction of the current flow, in other words the anodal or cathodal IP. In the case of the anodal IP with FD-4, the reduced TEER values in the conjunctiva recovered by 84% to the level before the current application up to 10 mA/cm^2 (Fig. 8B), while they recovered by 70% in the cornea up to 2.0 mA/cm^2 (Fig. 8A). The reduced TEER values in the anodal IP did not recover at 5.0 mA/cm^2 in the cornea and at 20 mA/cm^2 in the conjunctiva, respectively. In case of the cathodal IP with BA, a similar reduction and recovery of the TEER were observed in the cornea up to 2.0 mA/cm^2 (Fig. 9A) and in the conjunctiva up to 5.0 mA/cm^2 (Fig. 9B). The upper limit of the applied current against the TEER recovery seems to be better in the anodal IP. In addition, a delay in TEER recovery was evident at the applied current of 2.0 mA/cm^2 in the cornea and 10 mA/cm^2 in the conjunctiva. PD and I_{sc} also exhibited similar trend in recovery as TEER (Tables 2, 3).

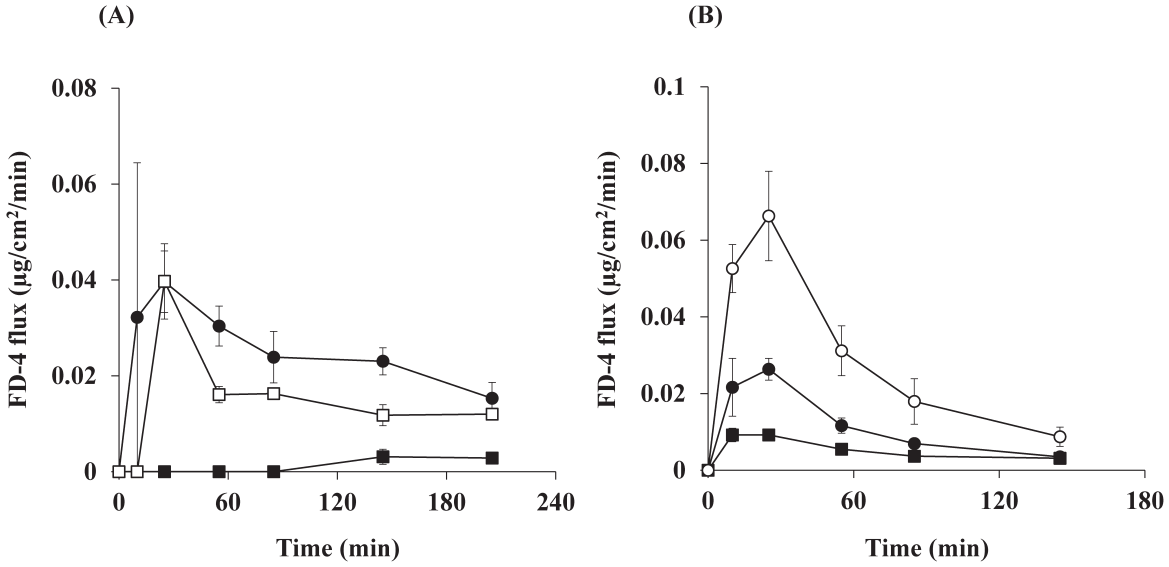

Fig. 7. Time–Courses of Released Flux of FD-4 from the Cornea (A) and Conjunctiva (B) after Anodal IP Application

■, 0 mA/cm^2; □, 0.5 mA/cm^2; ●, 2.0 mA/cm^2; ○, 10 mA/cm^2. Data represent the mean±S.E. (n=3–4).

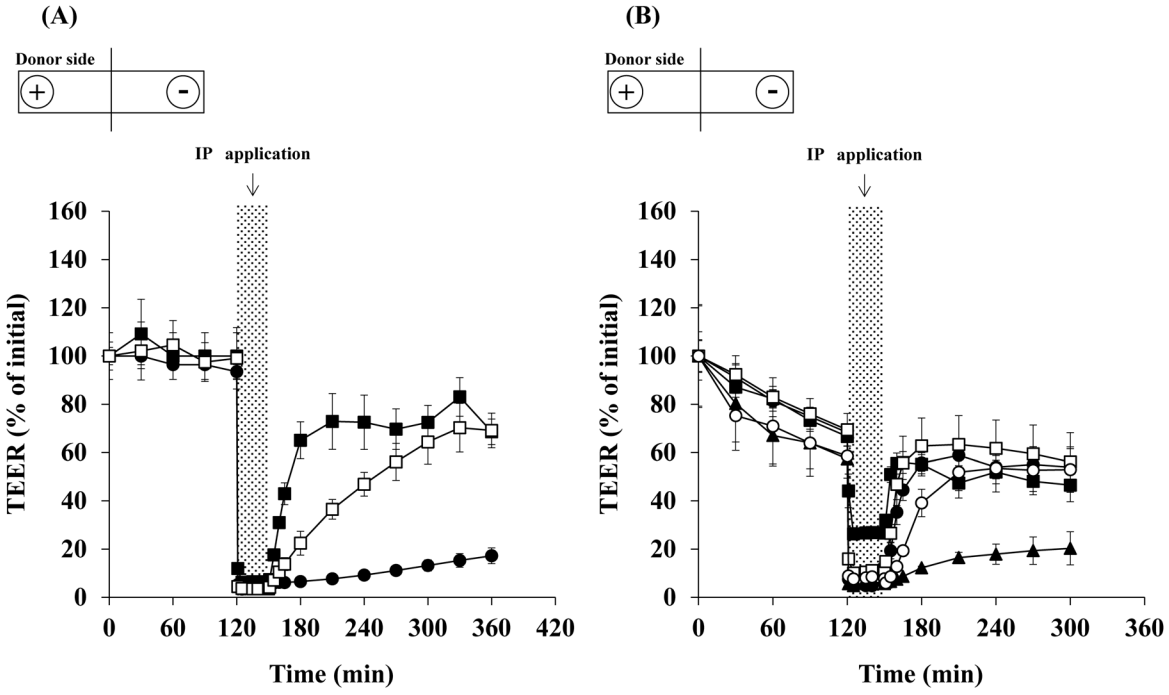

Fig. 8. Time–Courses of the Corneal (A) and Conjunctival (B) TEER in the FD-4 Permeation Experiments

■, 0.5 mA/cm^2; □, 2.0 mA/cm^2; ●, 5.0 mA/cm^2; ○, 10 mA/cm^2; ▲, 20 mA/cm^2. Data represent the mean±S.E. (n=3–8).

DISCUSSION

In the present study, we investigated the effect of electric current on the transport of ionic and non-ionic drug across the rabbit cornea and conjunctiva, as well as the viability and integrity of the tissues based on electrophysiological measurements.

Ocular *in vivo* IP studies were carried out using a relatively higher current density (0.8–28.2 mA/cm^2)[7–15] compared with that in the transdermal IP (*ca.* 0.5 mA/cm^2).[5] Therefore, in the case of IP in viable tissues, the potential influence of the applied electric current on the tissue functions in terms of viability and integrity could be concerned. However, influence of high electric current density on the viable tissues of cornea and conjunctiva has not been fully understood. The TEER values of the cornea and conjunctiva were drastically reduced by the application of both anodal and cathodal IP and recovered on cessation of the IP application as shown in Figs. 8 and 9. Because the TEER is known as an index of tissue integrity, application of the electric current to the ocular tissues has a marked effect on tissue integrity. In addition, a significant recovery of the tissue TEER was observed after cessation of the applied current at 2.0 mA/cm^2 in the cornea and 10 mA/cm^2 in the conjunctiva for the anodal IP and at 2.0 mA/cm^2 in the cornea and 5.0 mA/cm^2 in the conjunctiva for the cathodal IP. The recovery of PD and I_{sc} after the current application corresponded to the TEER recovery (Tables 2, 3), suggesting that

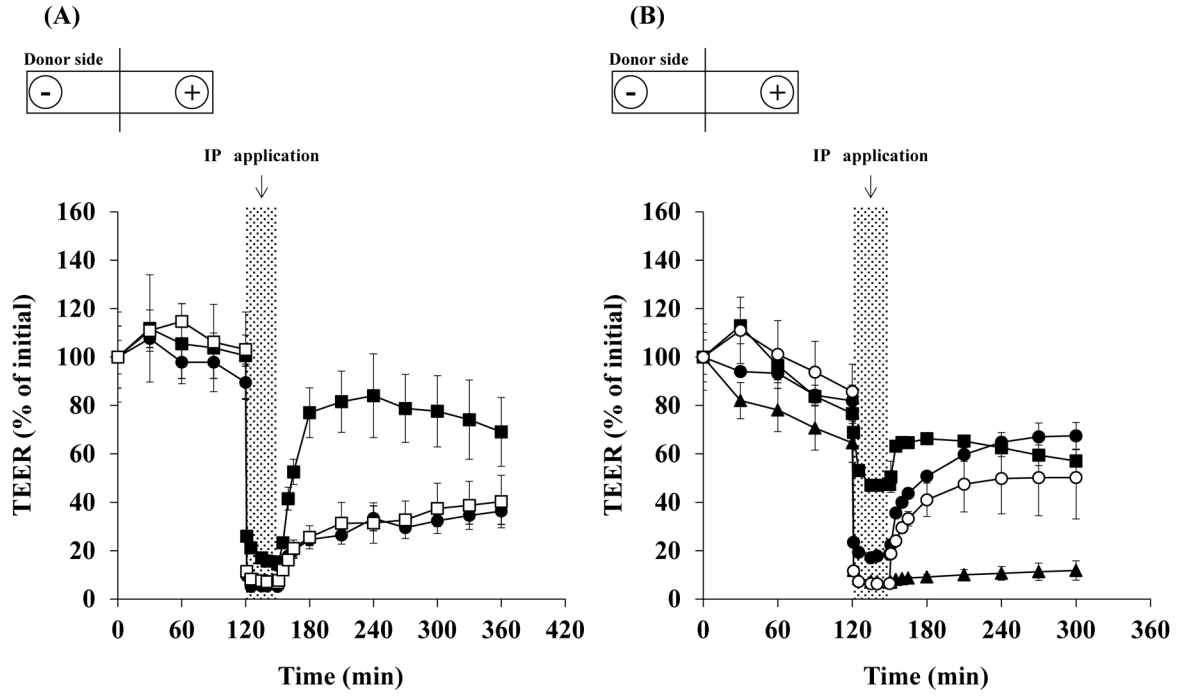

Fig. 9. Time–Courses of the Corneal (A) and Conjunctival (B) TEER in the BA Permeation Experiments

■, 0.5 mA/cm^2; □, 1.5 mA/cm^2; ●, 2.0 mA/cm^2; ○, 5.0 mA/cm^2; ▲, 7.5 mA/cm^2. Data represent the mean±S.E. (n=3).

Table 2A. PD and I_{sc} Values in the Cornea in the Anodal IP Experiments

	Current density (mA/cm^2)	Before IP	During IP	After IP (210 min)
[PD] (mV)				
	0.5	11.1±1.01	72.1±1.92	8.50±0.21
	2.0	11.1±2.31	157±26.0	6.97±1.65
	5.0	9.83±0.64	457±18.7	1.77±0.43
I_{sc} (μA/cm^2)				
	0.5	5.30±0.85	—	5.61±0.67
	2.0	5.30±1.21	—	4.70±1.29
	5.0	5.00±0.39	—	4.70±0.15

Data represent the mean±S.E. (n=3).

Table 2B. PD and I_{sc} Values in the Conjunctiva in the Anodal IP Experiments

	Current density (mA/cm^2)	Before IP	During IP	After IP (150 min)
[PD] (mV)				
	0.5	20.6±1.96	80.6±2.67	13.4±2.88
	2.0	23.1±4.30	115±17.1	13.8±3.35
	5.0	18.1±2.20	160±21.3	11.1±1.66
	10	22.6±3.33	444±54.5	10.9±1.61
	20	20.4±3.07	664±113	4.9±1.61
I_{sc} (μA/cm^2)				
	0.5	50.0±4.25	—	42.8±6.25
	2.0	62.0±11.2	—	46.6±8.89
	5.0	44.5±6.04	—	35.3±5.06
	10	60.8±6.49	—	35.3±4.81
	20	56.6±5.48	—	31.0±1.81

Data represent the mean±S.E. (n=3–8).

Table 3A. PD and I_{sc} Values in the Cornea in the Cathodal IP Experiments

	Current density (mA/cm^2)	Before IP	During IP	After IP (210 min)
[PD] (mV)				
	0.5	10.7±1.45	149±22.1	6.00±1.32
	1.5	10.8±0.76	217±29.1	2.60±0.61
	2.0	10.5±2.72	229±24.4	3.00±1.70
I_{sc} (μA/cm^2)				
	0.5	5.61±0.97	—	4.47±1.18
	1.5	4.70±0.08	—	3.41±0.13
	2.0	5.45±1.51	—	3.56±1.54

Data represent the mean±S.E. (n=3).

Table 3B. PD and I_{sc} Values in the Conjunctiva in the Cathodal IP Experiments

	Current density (mA/cm^2)	Before IP	During IP	After IP (150 min)
[PD] (mV)				
	0.5	15.9±1.65	118±1.06	12.2±2.02
	2.0	11.4±1.46	157±6.74	9.00±0.35
	5.0	15.0±4.44	158±11.8	6.93±3.58
	7.5	12.6±1.07	249±26.2	0.90±0.36
I_{sc} (μA/cm^2)				
	0.5	41.4±4.19	—	42.2±3.13
	2.0	35.9±1.85	—	33.9±2.80
	5.0	34.0±6.17	—	24.8±4.90
	7.5	42.5±4.21	—	16.1±2.71

Data represent the mean±S.E. (n=3).

the viability and integrity of the cornea and conjunctiva could be maintained after application of the electric current. In addition, the sensitivity to the applied current may be dependent on the current direction as the upper limit of the applied current for the recovery seems to differ between the anodal and cathodal IP. Moreover, the observed delay in the TEER recovery when a higher electric current was applied might be related to the tissue functions affected by the applied current. Further studies of the relationship between the applied current and cellular function of the tissues will help to establish the applied current conditions for safe ocular IP.

As shown in Figs. 1 and 2 and Table 1, LC and BA fluxes in the cornea and conjunctiva were increased in accordance with the application period of the anodal and cathodal IP. Such phenomena indicate that the transport of ionic drugs can be attributed to the flow of the electric current. Since the driving force of IP for ionic drugs is known the electrochemical potential gradient across the membrane,[33] it is supposed that transport of LC and BA is dependent on PD under the influence of the electric current. Indeed, the maximal fluxes achieved by the IP application were proportional to the corneal and conjunctival PD values during the IP application (Fig. 3). However, the enhancement ratios (approximately 2–4 fold) for LC and BA achieved by IP might not be noticeable compared with the typical cases obtained in transdermal IP.[34,35] Because a buffer solution such as BRS is required to maintain the tissue viability in the case of ocular tissues, ion species in BRS can affect the transfer of charges by ionic drugs across the cornea and conjunctiva under the influence of a constant current. Indeed, the transport numbers of LC and BA, ratio of movement of ionic species against the total electric current, under the conditions used in the present study calculated by the theoretical equation by Mudry *et al.*[36] were very low compared with those of coexisting ions such as Na^+ and Cl^- (data not shown). Such coexistence of a number of ion species in the drug solution may be disadvantageous for the IP enhancement of small molecular weight ionic drugs.

FD-4 fluxes across the cornea and conjunctiva were increased in a PD-dependent manner as shown Figs. 5A and B. Because the electroosmotic flow is proportional to potential difference as indicated by Pikal,[37] the observed increase in FD-4 (a hydrophilic macromolecule) fluxes in the cornea and conjunctiva by application of anodal IP (Fig. 4) are likely to be due to an electroosmotic effect, as aqueous solvent flow occurs in the direction of the anode to cathode in biological membranes.[37,38] The observed transient increase in the FD-4 flux in conjunctiva (Fig. 4B) possibly reflects transient induction of aqueous solvent flow by electroosmosis. Nevertheless, post-IP fluxes of FD-4 across the cornea showed a sustained increase as shown in Fig. 4A. Such sustained increase in the fluxes may be due to the potential retention of FD-4 in the cornea associated with the structural configuration of the cornea composed of multiple layers such as the epithelial layer, Bowman's layer, stroma, Dua's layer, Descemet's layer and the endothelial layer.[39,40] In fact, the amount of FD-4 in the cornea and accompanying release flux from the cornea at 210 min after IP application was significantly higher compared with the amount without IP application (Figs. 6A, 7A), suggesting that FD-4 migrated to the cornea with the assistance of IP and was retained inside tissues while neither significant retention nor sustained flux was observed in the case of the conjunctiva (Figs. 6B, 7B). Thus, the characteristics of the ocular IP are likely to be different between the cornea and conjunctiva with respect to the enhancement of large molecules. However, the corneal IP might involve difficulties since the enhanced fluxes of FD-4 in the cornea were much smaller (Table 1) and recovery of electrophysiological parameters of the cornea appears to be more sensitive to the applied current compared with the conjunctival IP (Tables 2, 3). Characterization of ocular IP demonstrated in the present study will provide useful information for ocular drug delivery using IP.

Application of IP in the cornea and conjunctiva induced a higher enhancing effect for FD-4 compared with LC and BA (Table 1) despite the restricted transport of FD-4 at baseline. It is known that large hydrophilic molecules such as FD-4 permeate *via* the paracellular pathway in the conjunctiva because the permeability coefficients of FD-4 are proportional to reciprocal of TEER (1/TEER).[27] In addition, FD-4 transport across the conjunctiva is increased in the presence of a paracellular modulator, poly-L-arginine (a polycationic enhancer) which induces the reversible reduction of the conjunctival TEER by affecting the localization of tight junction (TJ)-associated proteins.[41] As the IP application in the present study reversibly reduced the TEER in the cornea and conjunctiva (Figs. 8, 9), alteration in the paracellular transport pathway may possibly be induced by modulating the TJ functions when the electric current is applied, thereby increasing the transport of hydrophilic molecules *via* the paracellular pathway. In our previous pilot studies applying low electric current density in the excised rabbit conjunctiva,[42] application of anodal IP at 0.85 mA/cm^2 increased D-mannitol flux by 8.0-fold, FD-4 flux by 9.5-fold, FD-20 (20 kDa of FITC-labelled dextran) flux by 27.0-fold, and FD-70 (70 kDa of FITC-labelled dextran) flux by 57.5-fold, respectively, suggesting that IP in ocular tissues may achieve higher enhancement for compounds having more restricted paracellular permeability such as large molecules. However, change in paracellular pathway by modulation of TJ function under the influence of electric current is likely to be involved in such size-dependent enhancing effect of ocular IP. Further, studies in both cornea and conjunctiva with respect to TJ functions and molecular size under the influence of higher electric current density (*ca.* 20 mA/cm^2) will be required to understand the mechanistic aspect of IP enhancement in viable ocular tissues.

In conclusion, our *in vitro* study demonstrates that IP application with a constant current up to 2.0 mA/cm^2 for the cornea and 10 (anodal IP) or 5.0 (cathodal IP) mA/cm^2 for the conjunctiva can be used for the delivery of both ionic and non-ionic molecules into the eyes without any significant tissue damage as based on electrophysiological parameters. This technique may be useful especially for the delivery of large hydrophilic molecules. Because IP enables a superior drug delivery system with an ON/OFF control feature, such a system will be expected as an alternative to intraocular injection of large molecules.

Conflict of Interest The authors declare no conflict of interest.

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