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Research Article

Application of direct electric current to the corneal and conjunctival epithelia regulates the tight junctional assembly for ocular iontophoretic drug delivery

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Abstract

Objectives: In this study, we determined how iontophoresis (IP) affects tight junctions (TJs) in isolated rabbit corneas and conjunctiva.

Methods: Direct electric current in the range of 0.5–2.0 and 0.5–10 mA/cm^2 were applied to the cornea and conjunctiva, respectively, for 30 min. The localization and expression levels of TJ-associated proteins were assessed before and after the application of the electric currents using immunostaining and western blotting.

Results: In both corneal and conjunctival epithelia, the localization of proteins, such as claudin-1, claudin-4, occludin, and ZO-1, was temporarily altered by anodal and cathodal IP; however, the protein relocalization was slower at higher currents. Additionally, in both anodal and cathodal IP, the expression levels of claudin-1 and occludin in the cornea and conjunctiva remained unchanged after the application of the electric currents compared with those before.

Conclusion: Our results indicated that the application of a direct electric current temporarily regulated TJ assemblies without altering the levels of TJ-associated proteins in both the cornea and conjunctiva. This temporary weakening of the paracellular barrier by the current may be responsible for the enhanced drug transport across the cornea and conjunctiva induced by ocular IP.

Keywords: ocular drug delivery, iontophoresis, electric current, cornea, conjunctiva, tight junction

1. INTRODUCTION

Drug delivery to the eye remains challenging. When applied to ocular surface tissues, drugs spread in the tear film and must penetrate the cornea and conjunctiva/sclera to reach the eye [1]. During this process, only a small fraction of the drug enters the eye due to the high barrier properties of the corneal and conjunctival epithelia [2]. Therefore, innovative pharmaceutical approaches to improve drug delivery to the eye remain warranted.

Iontophoresis (IP) uses electricity to enhance drug absorption and has been extensively investigated to enhance drug delivery across the skin and mucosal surfaces, including ocular tissues [3,4]. *In vivo* studies have shown that ocular IP can transport higher amounts of certain drugs into the eye than eye drops or subconjunctival injections [5-13], which is especially promising for treating both anterior and posterior eye diseases since IP can target drugs in the aqueous and vitreous humors through the cornea and conjunctiva, respectively.

We have previously examined drug transport through IP using viable rabbit cornea and conjunctiva [14] and found that the permeability of fluorescein isothiocyanate dextran (FD-4), a 4.4-kDa hydrophilic macromolecule, across these tissues significantly increased when the anode was placed on the drug reservoir side (anodal IP). This increase in permeability was dependent on the applied electric current density. Additionally, anodal IP markedly reduced the transepithelial electrical resistance (TEER) of both the cornea and conjunctiva. The TEER recovered following the termination of IP application up to 2.0 mA/cm^2 for the cornea and 10 mA/cm^2 for the conjunctiva, although the recovery was slower at higher current densities. Nemoto *et al*. have reported that the application of poly-L-arginine (PLA), a polycationic absorption enhancer, to the conjunctiva increased the permeability coefficients of hydrophilic macromolecules and correlated with a decrease in TEER (1/TEER); the authors observed a reversible reduction in conjunctival TEER [15]. The treatment of polycationic enhancers boosts the paracellular permeability of hydrophilic compounds by opening tight junctions (TJs) at the cellular margins in epithelial tissue and cell layer [16-18]. Therefore, we hypothesize that electric current in ocular IP may temporarily enlarge the paracellular pathway by reversibly altering the assembly of TJ-associated proteins, thus enhancing the transport of hydrophilic compounds across the ocular tissues. Applied current density plays a crucial role in the assembly of TJ-associated proteins in the cornea and conjunctiva post-IP; however, the exact effect of iontophoretic electric currents on TJ function, particularly in regulating drug absorption, remains unclear.

Therefore, this study explores the effect of iontophoretic electric currents on the assembly of TJ-associated proteins in isolated rabbit corneal and conjunctival epithelia. focusing on specific TJ-associated proteins, namely claudin-1, claudin-4, occludin, and ZO-1. The localization and expression of these TJ-associated proteins were analyzed using immunostaining and western blotting, respectively, and were compared before and after the application of electric currents at different current densities. The potential effect of the current direction on the assembly was also assessed using anodal and cathodal IP, where the anode and cathode were placed in the drug reservoir.

2. MATERIALS AND METHODS

2.1 Chemicals

Mouse monoclonal anti-claudin-1 (Cat # 37-4900), anti-claudin-4 (Cat # 32-9400), anti-occludin (Cat # 33-1500), anti-ZO-1 (Cat # 33-9100), anti-GAPDH (Cat # AM4300), Alexa Fluor® 488 goat anti-mouse IgG, HRP-conjugated anti-mouse IgG, RNase A and propidium iodide were purchased from Thermo Fisher Scientific Inc. (Massachusetts, U.S.A.). All other chemicals were of special grade and used without further purification.

2.2 Animals

Male Japanese white rabbits, weighing between 2.5 to 3.5 kg, were obtained from Sankyo Labo Service Co., Inc. (Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University.

2.3 Preparation of cornea and conjunctiva

We prepared isolated rabbit cornea and conjunctiva as previously described [14]. Rabbits were anesthetized with a 20 mg/kg sodium pentobarbital solution injected into a marginal ear vein, and euthanized using a 3.3 M KCl solution injected at the same site. The entire eyeball was carefully removed from the orbit, and the cornea and conjunctiva were isolated, trimmed, and mounted meticulously onto a tissue adaptor with an effective circular area of 0.44 cm^2. This adaptor was placed between Ussing-type chambers maintained at 37 °C using a water jacket.

2.4 Electric current application

For low electric current (0.5–2.0 mA/cm^2), a short-circuit current measurement device (CEZ-9100, Nihon Kohden, Tokyo, Japan) was used. For a high electric current (5.0–10 mA/cm^2), an electric stimulator (SEN-8203, Nihon Kohden, Tokyo, Japan) with an isolator (SS-104J, Nihon Kohden, Tokyo, Japan) was used. In anodal IP, currents of 0.5 and/or 2.0 mA/cm^2 for the cornea and 0.5 and/or 10 mA/cm^2 for the conjunctiva were applied. For cathodal IP, currents of 0.5 and/or 2.0 mA/cm^2 for

the cornea and 0.5 and/or 5.0 mA/cm^2 for the conjunctiva were applied. Each current was applied for 30 min.

2.5 Immunostaining

The cornea and conjunctiva were fixed in acetone at −20 °C for 5 min and permeabilized with 0.1% Triton® X-100 for 10 min at room temperature. After blocking with 3% skim milk for 1 h at room temperature, tissues were incubated overnight at 4 °C with primary antibodies against claudin-1, claudin-4, occluding, and ZO-1 (1:40), followed by secondary antibody (Alexa® 488 goat anti-mouse IgG) for 2 h at room temperature. Nuclei were stained with RNase and propidium iodide. The samples were observed using a confocal laser scanning microscope (FV-1000, OLYMPUS, Tokyo, Japan).

2.6 Western blotting

Cornea and conjunctiva before and after electric current application were fractured in liquid nitrogen, homogenized in a radio-immunoprecipitation assay buffer, and centrifuged at 12,000 × *g* for 10 min at 4 °C. Protein samples from the supernatant were used for western blotting. Equal amounts of protein (30 μg/well for cornea, 40 μg/well for conjunctiva) were loaded onto 12% Mini-PROTEAN® TGX™ gel (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) and electrophoresed at 200 V for 35 min. Proteins were transferred onto a PVDF membrane at 70 V for 2 h. The membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies against claudin-1 (1:400), occludin (1:300), and GAPDH (1:40,000) overnight at 4 °C, followed by secondary antibody (HRP-conjugated anti-mouse IgG) for 2 hat room temperature. Chemiluminescence was detected using a ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc. California, U.S.A.). The pixel density of the TJ-associated proteins was analyzed using the ImageJ software.

2.7 Statistical analysis

Data were presented as the mean ± standard error (S.E.) from 4–6 experiments. Differences among group means were evaluated using the *Tukey–Klame*r test, and a *p-value* of < 0.05 was considered statistically significant.

3. RESULTS

3.1 Impact of electric current on TJ-associated protein localization in corneal and conjunctival epithelia

Figures 1 and 2 illustrate the time courses of the immunostaining images of TJ-associated proteins in the corneal epithelium under anodal and cathodal IP, respectively, while Figures 3 and 4 show similar images of conjunctival epithelium. In both anodal and cathodal IP, the fluorescence signals of claudin-1, claudin-4, occludin, and ZO-1 at the cellular margins of the cornea and conjunctiva (Figs. 1–4, before IP application) were reduced following the application of the electric current (Figs. 1–4, 0 min after IP application). The reduced signals of TJ-associated proteins were detectable at the cellular margins 210 min following current application to the cornea (Figs. 1 and 2, 210 min after IP application) and 90 min after IP application (Figs. 3 and 4, 90 min after IP application). Notably, under low electric current conditions (0.5 mA/cm^2), the reduced fluorescence signals of TJ-associated proteins reappeared at the cellular margins 30 (Figs. 1 and 2, 30 min after IP application) and 15 min after IP application (Figs. 3 and 4, 15 min after IP application). These observations suggest that the assembly of TJ-associated proteins is reversibly altered by the electric current, with the extent of the change depending on the current density.

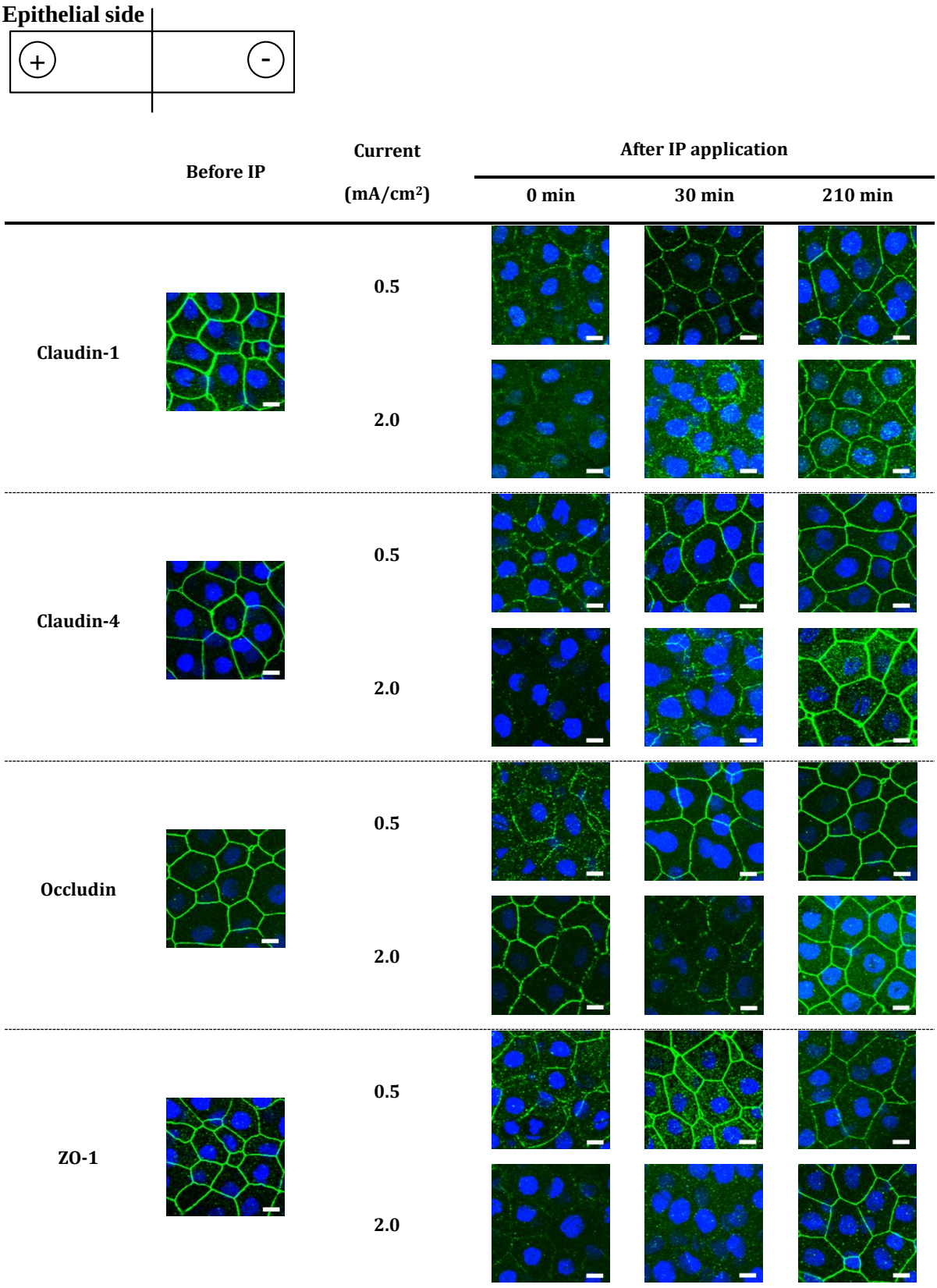

Figure 1: Localization of TJ-associated proteins in the rabbit corneal epithelium before and after anodal IP application. TJ-associated proteins in the corneal epithelium disappeared on the cellular margin following anodal IP application. After IP application, all proteins appeared on the cellular margin in a time-dependent manner. The recovery time of TJ-associated proteins was delayed at high electric current density (2.0 mA/cm²) compared with low current density (0.5 mA/cm²). Scale bar = 10 μm. Green fluorescence indicates TJ-associated proteins, while blue fluorescence represents nuclei.

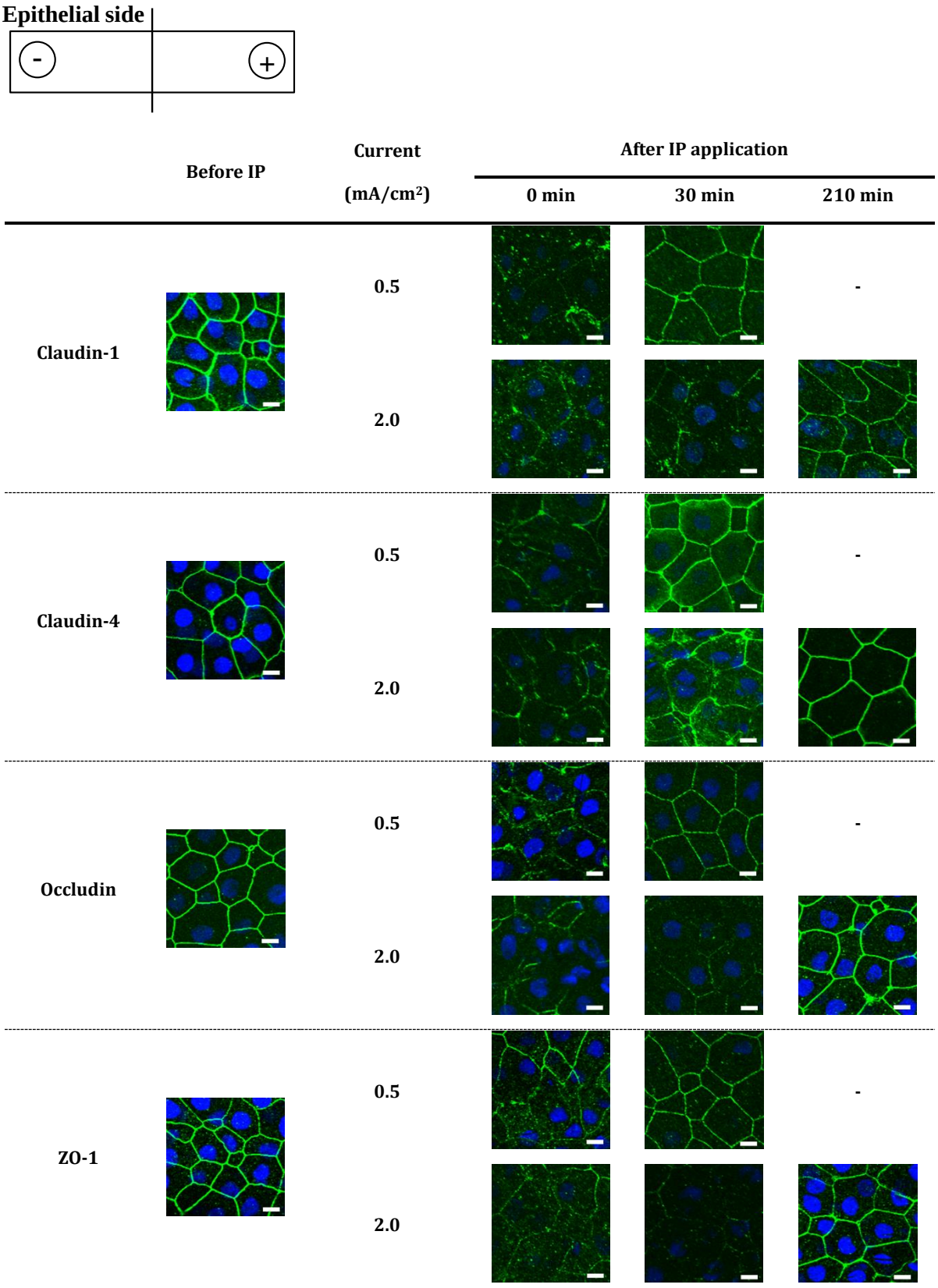

Figure 2: Localization of TJ-associated proteins in the rabbit corneal epithelium before and after cathodal IP application. TJ-associated proteins in the corneal epithelium disappeared on the cellular margin following cathodal IP application. After IP application, all proteins appeared on the cellular margin in a time-dependent manner. The recovery time of TJ-associated proteins was delayed at high electric current density (2.0 mA/cm²) compared with low current density (0.5 mA/cm²). Scale bar = 10 μm. Green fluorescence indicates TJ-associated proteins, while blue fluorescence represents nuclei.

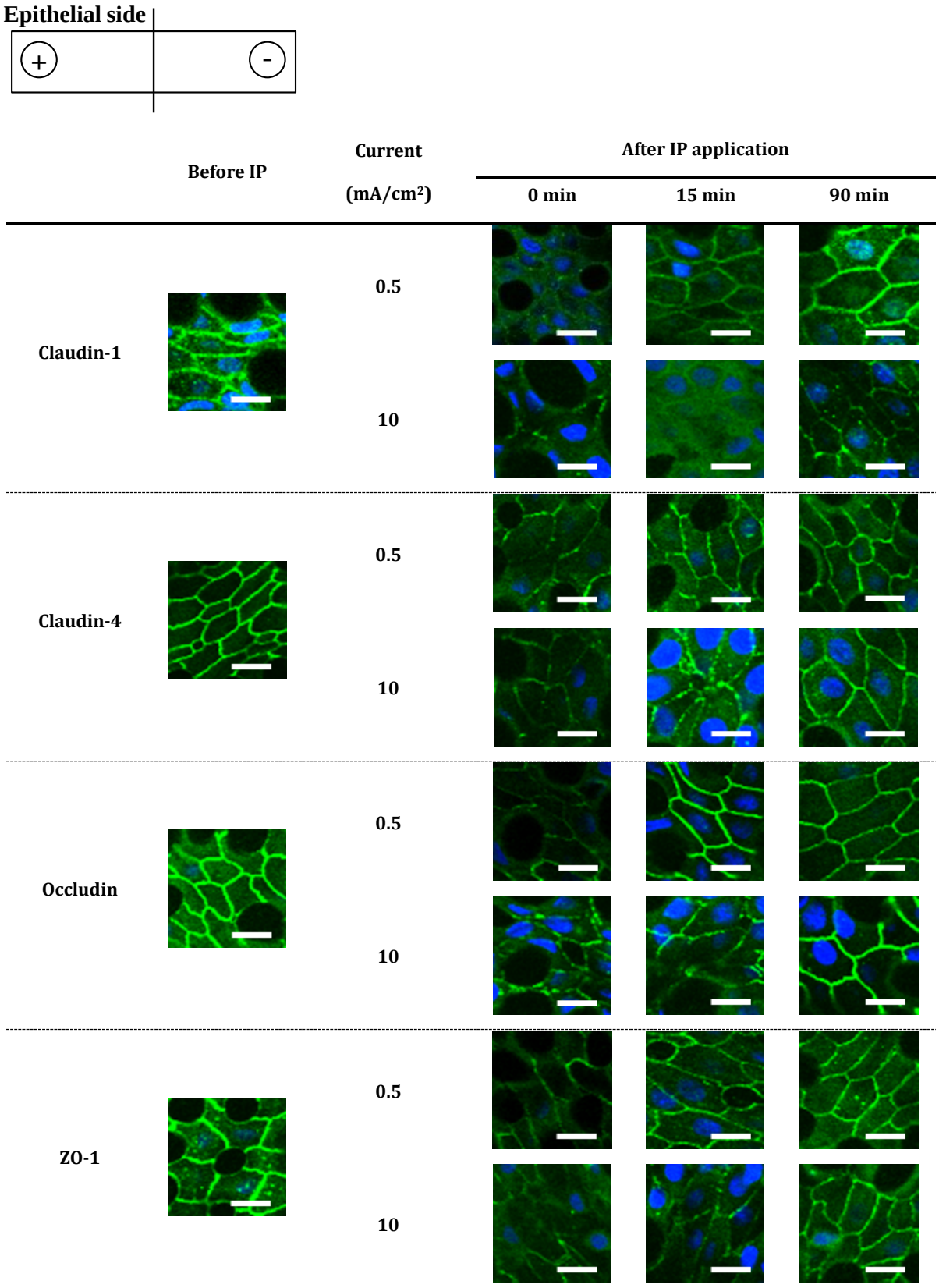

Figure 3: Localization of TJ-associated proteins in the rabbit conjunctiva epithelium before and after anodal IP application. TJ-associated proteins in the conjunctival epithelium disappeared on the cellular margin following anodal IP application. After IP application, all proteins appeared on the cellular margin in a time-dependent manner. The recovery time of TJ-associated proteins was delayed at high electric current density (10 mA/cm²) compared with low current density (0.5 mA/cm²). Scale bar = 10 μm. Green fluorescence indicates TJ-associated proteins, while blue fluorescence represents nuclei.

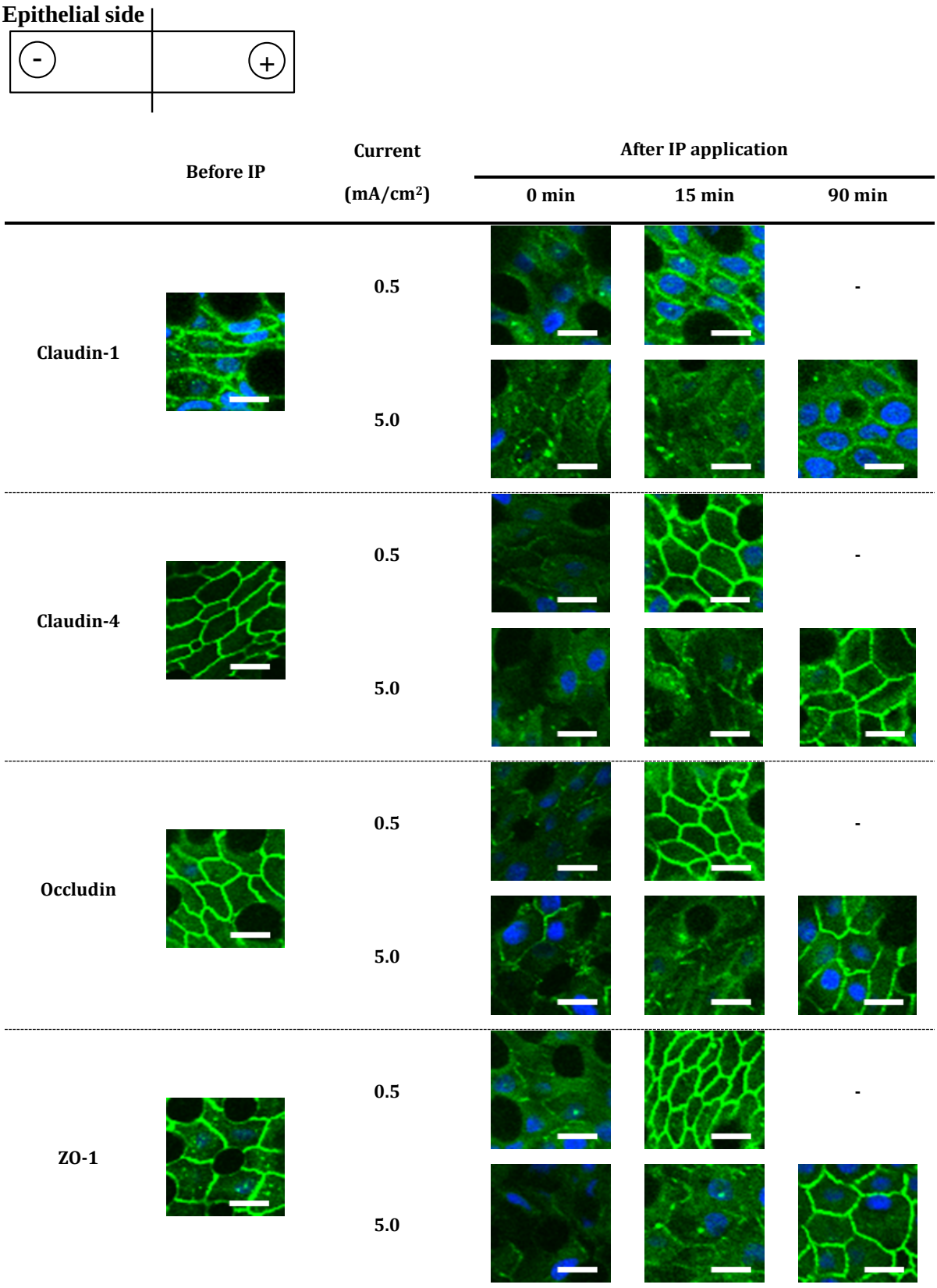

Figure 4: Localization of TJ-associated proteins in the rabbit conjunctiva epithelium before and after cathodal IP application. TJ-associated proteins in the corneal epithelium disappeared on the cellular margin following cathodal IP application. After IP application, all proteins appeared on the cellular margin in a time-dependent manner. The recovery time of TJ-associated proteins was delayed at high electric current density (5.0 mA/cm²) compared with low current density (0.5 mA/cm²). Scale bar = 10 μm. Green fluorescence indicates TJ-associated proteins, while blue fluorescence represents nuclei.

3.2 Analysis of claudin-1 and occludin expression after electric current application in cornea and conjunctiva

We next evaluated the expressions of claudin-1 and occludin in both the cornea and conjunctiva following the application of an electric current. Using both anodal and cathodal IP, the western blotting bands for claudin-1 and occludin showed similar patterns following current application (Figs. 5 and 6). The expression levels of claudin-1 and occludin at the end of current application were 1.01- and 0.95-fold, respectively, relative to their pre-application levels. Conversely, in the conjunctiva, the expression levels of claudin-1 and occludin at the end of current application were 1.37- and 0.92-fold higher than their initial levels, respectively. These findings suggest that IP application do not significantly affect the levels of claudin-1 and occludin. Moreover, neither the specific electric current densities (2.0, 5.0, and 10 mA/cm^2) nor the direction of the current (anodal and cathodal IP) significantly affected the expression levels of these proteins. This uniformity across various current densities and directions further corroborates that the application of electric current under the tested conditions did not substantially alter the levels of these proteins in the cornea and conjunctiva.

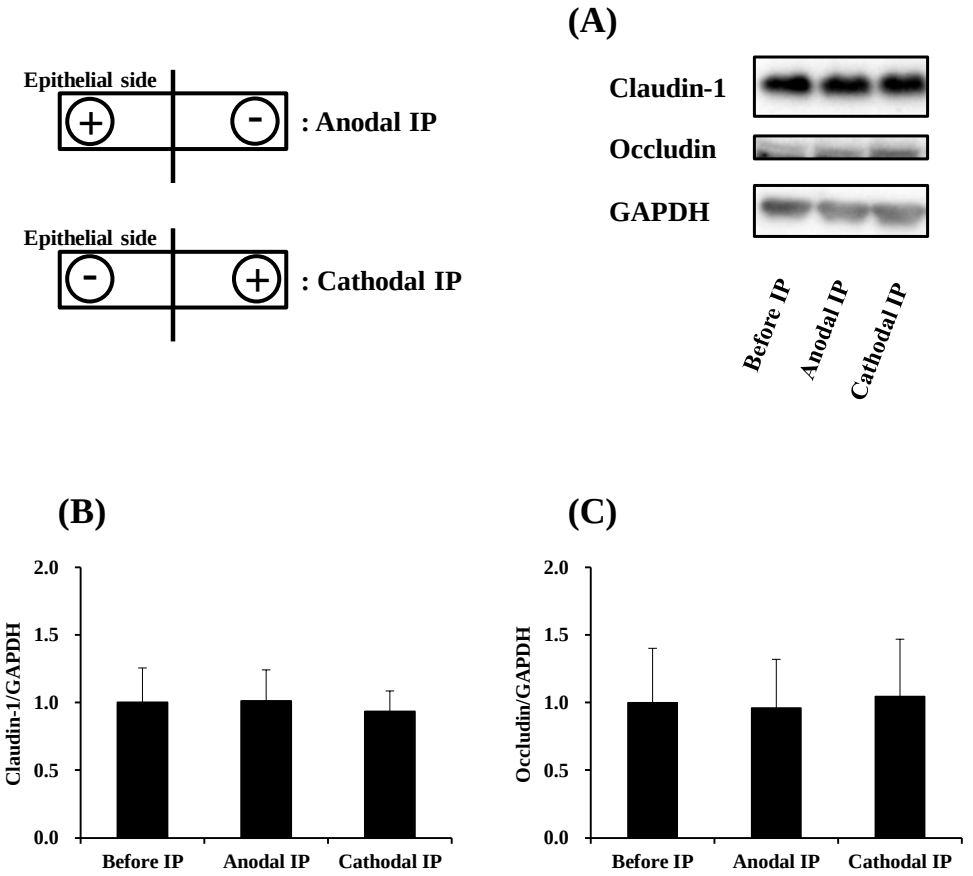

Figure 5: Expression of TJ-associated protein in the cornea before and termination of anodal (2.0 mA/cm^2) and cathodal (2.0 mA/cm^2) IP application. (A) Western blotting analysis in cornea; (B) and (C) the density of claudin-1 and occludin corrected to GAPDH, respectively. Data are shown as the mean ± S.E. (n = 6).

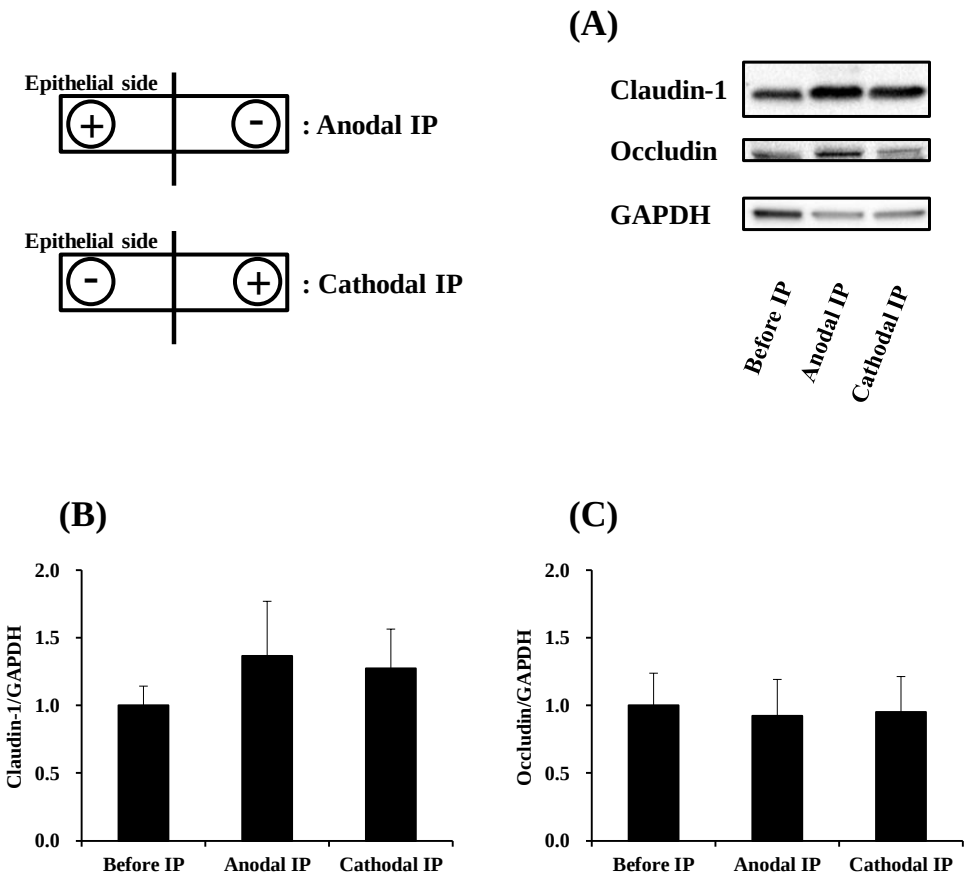

Figure 6: Expression of TJ-associated protein in the conjunctiva before and termination of anodal (10 mA/cm²) and cathodal (5.0 mA/cm²) IP application. (A) Western blotting analysis in conjunctiva; (B) and (C), the density of claudin-1 and occludin corrected to GAPDH, respectively. Data are shown as the mean ± S.E. (n = 4).

DISCUSSION

Differentiation of drug transport pathways in transdermal and ocular iontophoresis

In transdermal iontophoresis, extensively studied in IP research, the primary pathway for enhancing drug transport is through dermal appendages, such as hair follicles and sweat glands, where the electrical resistance is lower than that in the stratum corneum [19]. For ocular IP, such as corneal and conjunctival IPs, however, the electric current affects the paracellular spaces in the epithelium, which have lower electrical resistance than the transcellular pathway. This suggests that ocular IP enhances drug transport across the paracellular pathways. To use ocular IP effectively to enhance drug absorption into the eye, it is essential to elucidate the iontophoretic enhancement pathways across the cornea and conjunctiva for safe and effective application of ocular iontophoretic electric currents. Therefore, we investigated the impact of electric current on the localization and expression of TJ-associated proteins in viable corneal and conjunctival tissues.

TJ-opening dynamics in corneal and conjunctival epithelia induced by IP application

The localization of claudin-1, claudin-4, occluding, and ZO-1 in the corneal and conjunctival epithelium was reversibly altered by the application of both anodal and cathodal IP (Figs. 1–4). Moreover, the relocalization times of these proteins following current application varied between low (0.5 mA/cm²) and high (2.0, 5.0, and 10 mA/cm²) current densities (Figs. 1–4, after IP application). In our previous study, the TEER ratio after IP to before IP in both the cornea and conjunctiva indicated higher values within a relatively short time at low current density compared with high current density applications (Table 1) [14]. For instance, with anodal IP, the corneal TEER at 30 min following IP was 65.1 ± 7.67% for 0.5 mA/cm² and 22.7 ± 5.00% for 2.0 mA/cm² compared with the values before IP. However, TEER at the time of TJ-associated protein restoration (210 min) recovered to 68–69% irrespective of the current density. The TEER results [14], along with our current findings, suggest that ocular IP influences TJ assembly and the distribution of TJ-associated proteins in an electric current-dependent manner.

Furthermore, the disappearance of the fluorescence signals of TJ-associated proteins during current applications implies potential degradation or internalization within epithelial cells. Claudin-1 and occludin, known for their high barrier function at co-expressed sites in epithelial TJs, play crucial roles in forming TJs and limiting the paracellular permeability of hydrophilic macromolecules [20,21]. Consequently, variation in the intracellular levels of these proteins, influenced by IP, may enhance the permeation of hydrophilic macromolecules [14]. However, our analysis showed no significant difference in the expression of these proteins in the cornea and conjunctiva before and after current application (Figs. 5 and 6), suggesting rapid internalization and subsequent re-localization of TJ-associated proteins after IP application.

Table 1: Corneal and conjunctival TEER ratio of after to before IP calculated from the results of our previous ocular IP study [14]

Tissue	IP condition	Current density (mA/cm^2)	After IP application (min)	The TEER ratio of after IP to before IP (%)
Cornea				
	Anodal IP	0.5	0	6.22 ± 0.17
			30	65.1 ± 7.7
			210	68.6 ± 5.3
		2.0	0	3.57 ± 0.58
			30	22.7 ± 5.0
			210	69.8 ± 5.0
	Cathodal IP	0.5	0	14.4 ± 2.1
			30	63.8 ± 18.5
			210	68.7 ± 14.1
		2.0	0	5.83 ± 0.62
			30	27.5 ± 2.8
			210	40.6 ± 6.2
Conjunctiva				
	Anodal IP	0.5	0	46.7 ± 4.0
			15	79.4 ± 5.2
			90	78.0 ± 7.3
		10	0	13.1 ± 1.6
			15	33.0 ± 3.2
			90	92.8 ± 15.6
	Cathodal IP	0.5	0	64.2 ± 2.5
			15	84.3 ± 1.2
			90	81.5 ± 4.8
		5.0	0	7.48 ± 0.56
			15	45.2 ± 9.5
			90	58.2 ± 17.1

TEER ($\Omega \cdot cm^2$) before IP: ≥ 2,000 (cornea) and ≥ 400 (conjunctiva). Data are shown as the mean ± S. E. (n = 3–7).

Potential mechanism of TJ opening and transport enhancement by IP application

The absence of differences between anodal and cathodal IP in this study suggests that the direction of the electric current did not affect the TJ regulation mechanism. It has been reported that the TJ barrier is influenced by alterations in the physiological environment, such as ion concentration [22]. Since the electric current during IP application is the sum of cation and anion fluxes across biological membranes, extracellular ion mobility, such as Ca^{2+}, may contribute to the mechanism underlying TJ regulation. Additionally, physical forces, such as hydrostatic and osmotic pressure, as reported by Tokuda *et al*. in renal epithelial A6 cells, can influence the localization of claudin-1 [23,24]. Further studies on the influence of the extracellular environment under an electric field on TJ regulation are required to better understand the mechanism

underlying TJ regulation by electric current application.

In iontophoretic drug transport, drugs are physically driven by electro-repulsion for ionic drugs or electroosmosis for nonionic drugs [3]. Guy *et al.* reported that IP transport for large compounds is predominantly governed by electroosmosis under electric current application [25]. Since electric current application induces reversible changes in the localization of TJ-associated proteins in the corneal and conjunctival epithelium, it appears that the enhancing effect of IP on the drug transport of hydrophilic macromolecules across the ocular surface tissue contributes to the enlargement of the paracellular pathway by regulating TJs and electroosmosis caused by current application (Fig. 7).

In conclusion, this study demonstrated that electric current application to the cornea and conjunctiva induced the opening of paracellular spaces in epithelial tissues and that the opening duration is regulated by the applied electric current. The reversible opening of spaces by an electric current may contribute to the mechanism of intraocular drug delivery via ocular IP. Further investigations into, among others, the influence of iontophoretic physical forces on paracellular opening, remain warranted to determine the optimal conditions for ocular IP application.

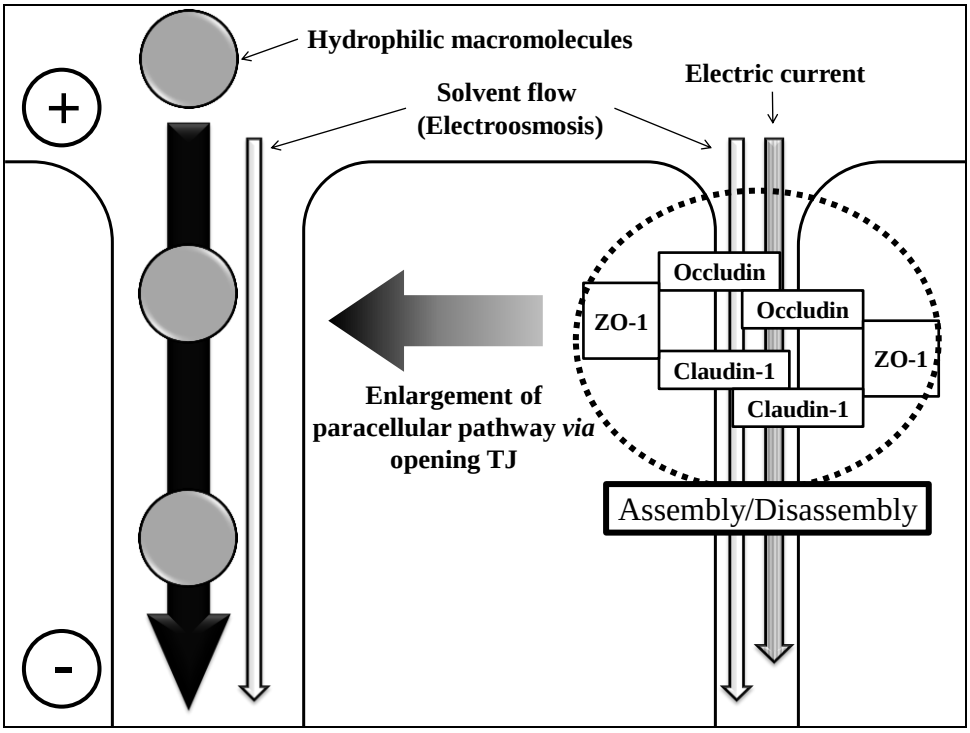

Figure 7: Proposed mechanism of iontophoretic transport of hydrophilic macromolecules through the cornea and conjunctiva. The application of IP to corneal and conjunctival epithelial cells expands the paracellular pathway, which is facilitated by the opening TJs driven by alterations in the assembly of TJ-associated proteins. This mechanism, combined with the electroosmotic flow occurring during IP application, is proposed to significantly enhance the passage of hydrophilic macromolecules across these ocular tissues.

Conflict of Interest

The authors declare no conflict of interest.

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