Transport of Timolol and Tilisolol in Rabbit Corneal Epithelium

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The purpose of this study is to characterize the transport of tilisolol and timolol through the corneal epithelium, which is believed to be a tight barrier of ocular drug absorption. Cultured normal rabbit corneal epithelial cells (RCEC) were used to investigate drug transport. Primary RCEC were seeded on a filter membrane of Transwell-COL® insert coated with fibronectin and grown in Dulbecco's modified Eagle's medium/nutrient mixture F-12 with various supplements. Beta-blocker permeability through the RCEC layer was measured to assess the transcellular permeability coefficient ($P_{transcell}$) in the absence or presence of inhibitors. The transcellular permeability of tilisolol was dependent on drug concentration although timolol showed no concentration dependency. Tilisolol flux from the apical to the basal side was larger than in the opposite direction although timolol showed no direction dependency. The transcellular permeability of tilisolol from the apical to the basal side was inhibited by sodium azide, tetraethylammonium, quinidine, taurocholic acid, guanidine and carnitine. Tilisolol had an active mechanism in uptake to the corneal epithelium, probably by the organic cation transporter family, although timolol predominantly permeated *via* passive diffusion. This RCEC system was useful to characterize the ocular permeation mechanism of drugs.

Key words cultured rabbit cornea; drug delivery system; transporter; beta-blocker; tilisolol; timolol

Beta-blockers decrease aqueous humor formation in the ciliary processes after instillation and are very often indispensable in the treatment of glaucoma.[1] However, most of the instilled amount is rapidly eliminated from the precorneal area and easily absorbed into the systemic circulation.[2] Beta-blockers in the precorneal area should also penetrate the tight barrier of the corneal epithelium into the eye.[3] Such behavior can result in poor bioavailability in the anterior segment and increase the severity of systemic adverse effects.[1—3] Many attempts have been made to deliver ophthalmic beta-blockers to the eye by means of different drug delivery systems.[4]

Ion transport processes have been extensively studied in the corneal epithelium.[5,6] The transport system of cationic and neutral amino acid in rabbit corneal epithelium and human cornea showed that this process was Na^+, Cl^-, and energy dependent.[7] On the other hand, a drug efflux pump, P-glycoprotein, was suggested to exist in the cornea epithelium and inhibit the corneal permeation of cyclosporine A.[8] Efflux pumps such as P-glycoprotein are believed to be a major barrier to drug delivery. Functional and molecular characterization showed the existence of P-glycoprotein in human cornea, rabbit cornea, and a rabbit corneal cell line.[9] There has, however, been little information about ophthalmic drug transporters in the cornea.

Recently, a few beta-blockers were suggested to be actively taken up by organic cation transporter in a transfected human cell line[10] and human renal brush-border membrane vesicles,[11] although many beta-blockers were thought to permeate through the cornea *via* passive diffusion.[12,13] The uptake of beta-blockers by a transporter into the cornea may be useful for targeting drugs into the eye and for reducing the instilled amount. The cornea consists of five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. Stratified epithelial cells with tight junctions are considered to comprise the corneal penetration barrier. Kawazu *et al.*[13] established an *in vitro* cultured normal rabbit corneal epithelial cell (RCEC) system to investigate transcellular drug permeation. In our study, we characterize the transport of beta-blockers, tilisolol and timolol, through the corneal epithelium using the RCEC system. Tilisolol is a beta-blocker that showed concentration-dependent flux in the preliminary experiment. Timolol is one of the most frequently prescribed drugs for glaucoma.

MATERIALS AND METHODS

Materials and Animals FITC-dextran (FD-4, MW 4400) and 6-carboxyfluorescein (6-CF) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Sodium azide (NaN_3), 2,4-dinitrophenol (DNP), taurocholic acid sodium salt (TA), tetraethylammonium chloride (TEA), quinidine sulfate (QUI), L-carnitine hydrochloride (CAR), guanidine hydrochloride (GUA) and verapamil hydrochloride (VER) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Timolol maleate was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Tilisolol was kindly supplied by Nisshin Flour Milling Co. Ltd. (Tokyo, Japan). D-[1-^{14}C]-Mannitol (specific activity, 2.11 GBq/mmol) was purchased from Amersham Life Science (Buckinghamshire, U.K.). All other chemicals were commercial products of reagent grade.

Primary cultured cells were obtained from Kurabo Industries Ltd. (Osaka, Japan). Transwell-COL® cell culture chambers (pore size 0.4 μm, diameter 12 mm, surface area 1 cm^2) were purchased from Costar (Bedford, MA, U.S.A.). Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12), fetal bovine serum (FBS) and other culture reagents were from GIBCO (Grand Island, NY, U.S.A.). Epidermal growth factor (EGF), choleratoxin (CTX), hydrocortisone (HCS) and insulin-transferrin sodium selenite media

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supplement (ISL) were from Sigma Chemicals. Penicillin G and streptomycin were from Wako Pure Chemical Industries Ltd. Human fibronectin was from Boehringer Mannheim GmbH (Mannheim, Germany).

Cell Culture RCECs were cultured according to the standard method reported previously.[13] RCECs 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 antibiotics (penicillin G 100 IU/ml+streptomycin 100 μg/ml). The Transwell-COL®insert was pre-coated with 4.0 μg human fibronectin as the attachment factor at room temperature for 30 min. RCECs were seeded at a density of 4×10^4 cells/cm^2 on the filter membrane of the Transwell-COL® insert and cultured at 37 °C under 95% air and 5% CO_2. The culture medium was replaced every day. The barrier of the RCEC layer grown on the filter membrane was assessed by measuring transepithelial electrical resistance (TEER) with a Millicell ERS electrical resistance meter (Millipore, Bedford, MA, U.S.A.) at different time points after seeding. The integrity of the cell layer was checked at the beginning and end of permeability experiments by determining the TEER.

Permeability Study Using RCEC In the permeability study, RCECs grown on a filter membrane were washed three times with Hank's balanced salt solution (HBSS) (1.3 mM $CaCl_2$, 5.0 mM KCl, 0.3 mM KH_2PO_4, 0.8 mM $MgCl_2$, 138 mM NaCl, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose and 10 mM HEPES for pH 7.4) and preincubated for 30 min at 37°C in a 5% CO_2 atmosphere before permeability experiments.

Drug permeability from the apical to the basal side (A-to-B) was initiated by removing all HBSS on the apical side (0.5 ml) and replacing it with HBSS containing various concentrations of drugs at 37 °C. At 30, 60, 90, and 120 min, a sample (0.9 ml) was collected from the basal side (1.5 ml) and was replaced with an equal volume of HBSS. Drug permeability from the basal to the apical side (B-to-A) was also initiated by replacing with drug solution on the basolateral side, and a sample (0.3 ml) was collected from the apical side at 37 °C. The samples were used for drug determination with high performance liquid chromatography (HPLC). The hydrophilic compounds used were [^{14}C] mannitol (1.85 kBq), 6-CF (50 μM), and FD-4 (50 μM). Tilisolol (50, 500, 5000 μM) and timolol (50, 500, 5000 μM) were used as models of ophthalmic beta-blockers. In order to examine the temperature effect, transport experiments for tilisolol and timolol were carried out at 4 °C. Drug permeability through a filter membrane without the cell layer was also examined in the same manner.

In the permeability study with inhibitors, the cell layer was preincubated with inhibitors on both apical and basal sides for 30 min. The inhibitor was also present on both sides during the experiment. Inhibitors including NaN_3 (10 mM), DNP (200 μM), TA (200 μM), TEA (200 μM), QUI (200 μM), CAR (200 μM), GUA (200 μM), and VER (200 μM) were used.

Calculation of Transcellular Permeability Coefficient The apparent permeability coefficient through overall membrane (P_{app}, cm/s) was calculated from the slope (flux, nmol/h) of the drug amount *vs.* the time profile on the receiver side (P_{app}=slope/3600/surface area of the layer/initial concentration on the donor side).

The permeability coefficient of drugs through the filter membrane (P_{filt}) was obtained from drug flux through the fibronectin-coated filter membrane. The permeability coefficient of drugs through the RCEC layer (P_{cell}) was calculated by Eq. (1).

$$P_{cell}=P_{app}\times P_{filt}/(P_{filt}-P_{app}) \quad (1)$$

Further, P_{cell} includes the permeability coefficients *via* the transcellular route ($P_{transcell}$) and the paracellular route ($P_{paracell}$).

$P_{transcell}$ was calculated by subtracting $P_{paracell}$ from P_{cell}. Hydrophilic drugs mainly permeate through the paracellular route because they cannot distribute into the cell. P_{cell} of hydrophilic drugs means $P_{paracell}$. The $P_{paracell}$ of tilisolol and timolol was calculated from the inverse relationship between P_{cell} and the square root of the molecular weight in hydrophilic compounds.

Drug Determination The sample of 6-CF and FD-4 was assayed using a spectrofluorophotometer (EP-770, Spectroscopic Co., Ltd., Japan; excitation and emission wavelengths; 492 and 524 nm for 6-CF; 495 and 514 nm for FD-4). Samples of [^{14}C] mannitol were measured using a liquid scintillation counter (Tri-Carb® Models 2100TR, Packard Co., Meriden, CT, U.S.A.).

Tilisolol and timolol were determined by HPLC. Samples of tilisolol and timolol (0.3 ml) were mixed with methanol (0.6 ml), including an internal standard (0.363 μM *o*-ethoxybenzamide for tilisolol and 0.05 mM phenacetin for timolol). The mixture was centrifuged at 12000 ***g*** for 10 min and 50 μl of supernatant was injected into an HPLC system (LC-10AD, Shimadzu Co., Ltd., Kyoto) in reverse-phase mode. The stationary phase used was a Cosmosil® 5C_{18}-MS packed column (150 mm length×4.6 mm i.d., Nacalai Tesque Inc.). A mixture of acetonitrile and 10 mM KH_2PO_4 (85 : 15 v/v) was used as the mobile phase with a flow rate of 1.0 ml/min. Drugs were monitored with a fluorescence detector (RF-10A, Shimadzu Co. Ltd.; excitation and emission wavelengths; 315 nm and 420 nm) for tilisolol and a UV spectrophotometric detector (SPD-10A, Shimadzu Co. Ltd.; wavelength; 290 nm) for timolol.

RESULTS

Using DMEM/F-12 containing 5% FBS, supplemented with EGF, CTX, ISL, and HCS, RCEC were readily attached to the matrix and began to spread. The density of seeding was 4×10^4 cells/cm^2 and led to rapid confluence. When allowed to become highly postconfluent, the cells became very tightly packed, and stratified on the membrane surface. The cells formed multilayers and had high transparency. TEER increased to the maximum around day 8 after inoculation. Peak TEER was 143.8±23.3 ohm×cm^2. Cultured cells appeared to be more closely approximate the morphology of *in vivo* corneal tissue.

Table 1 shows the values of P_{filt}, P_{cell}, and $P_{paracell}$ of hydrophilic compounds and beta-blockers. The values of P_{filt} and P_{cell} were not significantly different between A-to-B and B-to-A directions. P_{filt} values of hydrophilic compounds were much larger than P_{cell} values. There was a linear relationship between the square root values of molecular weight and P_{cell} values in hydrophilic drugs, which mainly permeate through the paracellular route. The P_{cell} of hydrophilic drugs means

Table 1. Permeability Coefficients (P_{filt}, P_{cell}, and $P_{paracell}$) of Hydrophilic Compounds and Beta-Blockers through Filter Membrane and RCEC Layer in Different Directions

Drug	P_{app} ($\times 10^{-6}$ cm/s)		P_{filt} ($\times 10^{-4}$ cm/s)		P_{cell} ($\times 10^{-6}$ cm/s)		$P_{paracell}$ [a] ($\times 10^{-6}$ cm/s)	
	A-to-B	B-to-A	A-to-B	B-to-A	A-to-B	B-to-A	A-to-B	B-to-A
^{14}C-Mannitol	4.35	3.99	1.34	1.58	4.49	4.09	4.49	4.09
6-CF	2.21	2.17	1.33	1.37	2.25	2.21	2.25	2.21
FD-4	0.83	0.94	0.54	0.51	0.84	0.92	0.84	0.92
Tilisolol	6.30	4.49	1.06	1.04	6.71	4.70	2.73	2.66
Timolol	13.70	9.17	0.71	0.65	17.04	10.68	2.46	2.43

Each value represents the mean of at least 3 experiments. Experiments were carried out at the concentration of 50 μM for 6-CF, FD-4, tilisolol and timolol and 1.75 μM for ^{14}C-Mannitol (18.5 kBq). A-to-B: Apical side to basal side direction. B-to-A: Basal side to apical side direction. *a*) $P_{paracell}$ for beta-blockers was calculated based on the relationship between the P_{cell} values of hydrophilic compounds and molecular weights.

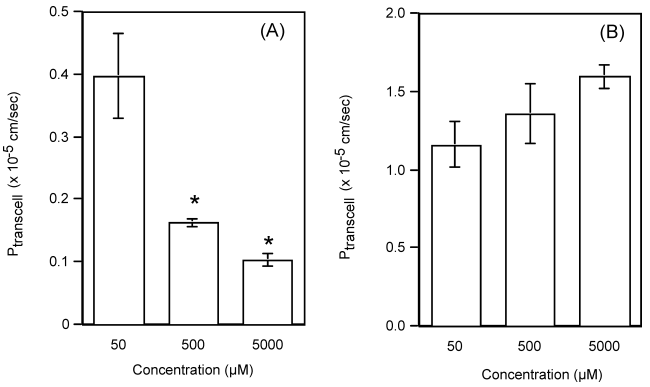

Fig. 1. Effect of Drug Concentrations on Permeability Coefficients ($P_{transcell}$) of Tilisolol (A) and Timolol (B) through the RCEC Layer in the Apical to the Basal Side (A-to-B) Direction

Data represent the average of at least three experiments±S.E. (*$p<0.05$: significantly different from $P_{transcell}$ of 50 μM by Student's *t*-test).

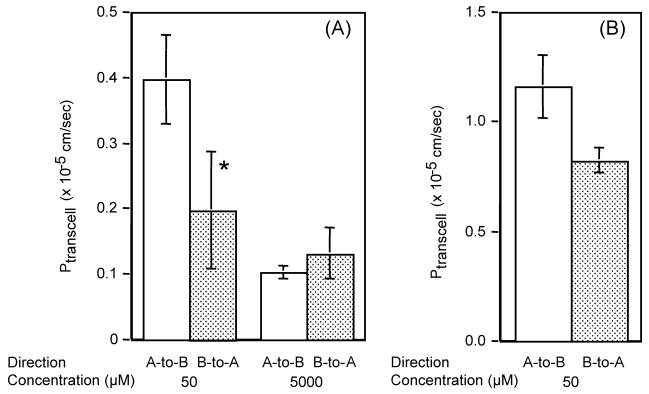

Fig. 2. Effect of Directions on Permeability Coefficients ($P_{transcell}$) of Tilisolol (A) and Timolol (B) through the RCEC Layer at 50 or 5000 μM

A-to-B: Apical to basal side direction. B-to-A: Basal to apical side direction. Data represent the average of at least three experiments±S.E.

$P_{paracell}$. Based on this relationship, the $P_{paracell}$ values of beta-blockers were calculated from their molecular weights.

The $P_{transcell}$ values of tilisolol and timolol were calculated from the permeation profiles in the A-to-B direction at various concentrations (50, 500, 5000 μM) and are presented in Fig. 1. The $P_{transcell}$ values of tilisolol significantly decreased with an increase of drug concentrations although timolol showed almost constant values regardless of drug concentrations. Figure 2 shows the $P_{transcell}$ values of tilisolol and timolol (50 μM) in different directions. The $P_{transcell}$ value of tilisolol in the A-to-B direction was significantly larger than that in the opposite direction (B-to-A) at 50 μM although there was no significant difference in both A-to-B and B-to-A directions at 5000 μM. Timolol showed no significant difference at 50 μM in both A-to-B and B-to-A directions.

As a result of transport experiments at 4 °C, the transcellular transport of tilisolol was not detected at 4 °C in the A-to-B direction although the $P_{transcell}$ value of timolol decreased to 12.4±1.5% (the average of at least three experiments±S.E.) of that at 37 °C. Figure 3 shows the effect of metabolic inhibitors such as NaN_3 and DNP on the $P_{transcell}$ values of tilisolol and timolol (50 μM) in the A-to-B direction. NaN_3 and DNP significantly reduced the $P_{transcell}$ values of tilisolol. The $P_{transcell}$ value of timolol was not significantly influenced by NaN_3. DNP was not used in the permeation of timolol because it influenced the assay. Figure 4 shows the effect of substrates as transporters as competitive inhibitors of $P_{transcell}$ values of tilisolol and timolol (50 μM) in the A-to-B direction. The $P_{transcell}$ values of tilisolol were significantly suppressed by TEA, TA, QUI, GUA, CAR, and VER, although TEA did not significantly inhibit the permeability of timolol.

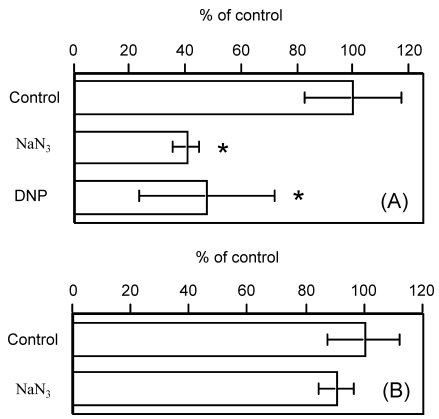

Fig. 3. Effect of Metabolic Inhibitors on Permeability Coefficients ($P_{transcell}$) of Tilisolol (A) and Timolol (B) through the RCEC Layer at 50 μM in the A-to-B Direction

Control is the $P_{transcell}$ at 50 μM in the apical to basal side (A-to-B) direction. Data represent the average of at least three experiments±S.E. (*$p<0.05$: significantly different from control by Student's *t*-test).

DISCUSSION

Generally, topical application of a drug is the method of choice because of its convenience and safety for ophthalmic

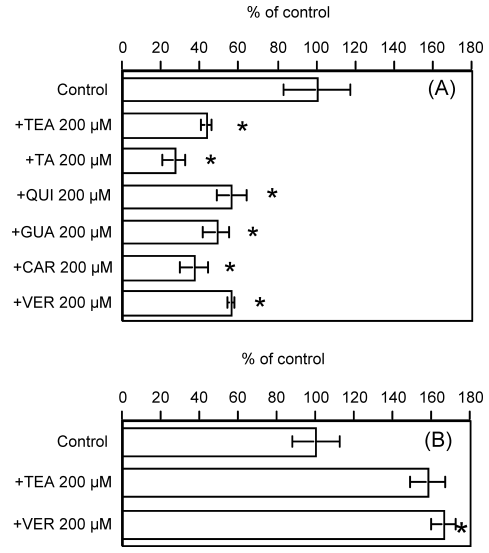

Fig. 4. Effect of Transporter Substrates on Permeability Coefficients ($P_{transcell}$) of Tilisolol (A) and Timolol (B) through the RCEC Layer at 50 μM in the Apical to Basal Side (A-to-B) Direction

Control is the $P_{transcell}$ at 50 μM in the A-to-B direction. Data represent the average of at least three experiments±S.E. (* $p<0.05$: significantly different from control by Student's *t*-test).

chemotherapy. The cornea is considered to be a major pathway for ocular permeation of topically applied drugs.[2,3] However, the outer epithelium of the cornea, which is composed of superficial layers of flat, tightly fitted squamous cells, provides the greatest resistance to drug permeation.[12] The estimated shunt resistance in the intact full-thickness cornea is 12—16 kohm $\times$cm^2 for normal cornea.[14] The permeability of ^{14}C-mannitol, 6-CF, and FD-4 of the RCEC layer was much higher than for the whole cornea[15] because of a lower TEER in this culture system. On the other hand, the transcellular permeability of timolol and tilisolol ($P_{transcell}$ values) almost agreed with their permeability coefficients through an excised cornea, as reported previously[16,17] (corneal permeability coefficient: 1.23×10^{-5} cm/s for timolol and 0.272×10^{-5} cm/s for tilisolol). This agreement indicates that beta-blockers predominantly permeate *via* the transcellular route across the tight cornea. Despite leakage in this model system, it is still appropriate for evaluating transcellular drug permeability. We investigated further mechanisms of transcellular transport of tilisolol and timolol across the RCEC layer.

Beta-blockers are widely used in the clinic to treat diseases related to the cardiovascular system and ocular hypertension, glaucoma.[1] They represent a family of compounds with a wide range of lipophilic properties. Tilisolol is a nonselective hydrophilic beta-blocker and reduced intraocular pressure after instillation in the rabbit eye.[18] Epithelial permeability of tilisolol was direction- and concentration-dependent, indicating the existence of specific uptake systems in apical to basal transport (Figs. 1, 2). It was important to note that the $P_{transcell}$ value for tilisolol decreased much more with decreased temperature (4 °C) than that for timolol because decreased temperature highly reduces the activity of active transport. The specific permeability of tilisolol in the A-to-B direction was significantly decreased by metabolic inhibitors, NaN_3 and DNP (Fig. 3). These results showed that the uptake of tilisolol was mediated by an active transporter. Active transport is an energy-dependent process characterized by solute movement against a chemical potential gradient. In general, the permeability of various organic cations was mediated by various types of organic cation transporters (OCT) and multidrug and toxin extrusion (MATE), which is the most recently classified multidrug resistance-conferring protein family, in the liver and kidney.[19—22] TEA was transported by OCT1, OCT2, OCTN1, MATE[10,11,20—24] and QUI was transported by OCT1.[25] TA was transported by a sodium-dependent bile acid transporter,[26,27] GUA was transported by OCT1[11] and CAR was transported by OCTN2.[28] VER was a substrate for the transport by P-glycoprotein and also significantly suppressed the transport of TEA and GUA.[10,11] Tilisolol permeability was significantly reduced by TEA, QUI, GUA, CAR and VER (Fig. 4). These results suggested the contribution of the OCT family or MATE family to the active transport of tilisolol in the corneal epithelium. Significant decrease of tilisolol transport by TA might cause the formation of micelles or a complex with tilisolol because of its surfactant activity and negative charge, although the TA inhibition mechanism needs further clarification.

Another nonselective beta-blocker, timolol, is one of the most frequently prescribed drugs for glaucoma. The epithelial permeability of timolol was neither direction- nor concentration-dependent and was not influenced by a metabolic inhibitor, NaN_3 (Figs. 1, 2, 3). These results showed that timolol predominantly permeated by passive diffusion. Passive diffusion is an energy-independent process characterized by solute movement in response to a chemical potential gradient. However, timolol permeability was partially influenced by temperature and VER. These results might indicate the contribution of special transport to timolol permeability in RCEC.

Thus, tilisolol showed active uptake, probably by organic cation transporter or multidrug and toxin extrusion transporter, although timolol seemed to be predominantly permeated by passive diffusion. There have been no reports about drug permeability by these cation transporters in the corneal epithelium. Further study is necessary to examine the contribution of the transporter to the corneal permeability of timolol. The RCEC system is useful to characterize the drug transport mechanism through the corneal epithelium.

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