Role of P-Glycoprotein in Restricting Propranolol Transport in Cultured Rabbit Conjunctival Epithelial Cell Layers

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Purpose. To determine the role of P-glycoprotein (P-gp) in propranolol transport in cultured rabbit conjunctival epithelial cell layers (RCEC).
Methods. The localization of P-gp in the cultured RCEC as well as in the excised conjunctiva was determined by immunofluorescence technique. The role of P-gp in transepithelial transport and uptake of propranolol in conjunctival epithelial cells cultured on Transwell filters was evaluated in the presence and absence of P-gp competing substrates, an anti-P-gp monoclonal antibody (4E3 mAb), or a metabolic inhibitor, 2, 4-dinitrophenol (2,4-DNP).
Results. Immunofluorescence studies revealed positive staining in the apical membrane of cultured RCEC and in the apical surface of the superficial cell layers in the excised conjunctiva, but not the basolateral membrane of cultured RCEC. Transport of propranolol showed preference in the basolateral-to-apical direction. The net secretory flux was saturable with a K_m of 71.5 ± 24.0 nM and a J_{max} of 1.45 ± 0.17 pmol/cm^2/hr. Cyclosporin A, progesterone, rhodamine 123, verapamil, 4E3 mAb and 2,4-DNP all increased apical 50 nM propranolol uptake by 43% to 66%. On the other hand, neither β-blockers (atenolol, metoprolol, and alprenolol) nor organic cation transporter substrates (tetraethylammonium (TEA) and guanidine), affected apical 50 nM propranolol uptake.
Conclusions. The energy-dependent efflux pump P-gp appears to be predominantly located on the apical plasma membrane of the conjunctival epithelium. It may play an important role in restricting the conjunctival absorption of some lipophilic drugs.

KEY WORDS: P-glycoprotein; conjunctival transport; drug efflux; cell culture model; propranolol.

INTRODUCTION

P-glycoprotein (P-gp) is a 170 kDa membrane protein encoded by the multidrug resistance gene (MDR1) and functions as an energy-dependent efflux pump (1). P-gp is typically localized at the apical surface of the epithelial cells and has been shown to reduce the transepithelial permeation of diverse drugs, including cyclosporin A (CsA) (2,3), vinblastine (4), daunomycin (5), etoposide (6), cimetidine (7) and celiprolol (8). High levels of P-gp expression have been found in human adrenal cortical cells, the brush border of renal proximal tubule epithelium, and the lumenal surface of biliary hepatocytes, small and large intestinal mucosal cells, and pancreatic ductules (9). In the eye, P-gp has been found in the conjunctiva (3), cornea (10), iris (11), and retina (11).

P-gp has been implicated in the secretion of a β-blocker (celiprolol) in an intestinal epithelial cell line (Caco-2) (8). In addition, uptake of β-blockers (acebutolol, celiprolol, nadolol, and timolol) by multidrug-resistant leukemic cell line variant K562/ADM was 37–63% lower than that by drug-sensitive K562 cells, indicating that the transport of β-blockers may be affected by P-gp (4).

The main objective of the present study was to determine the role of P-gp in modulating the transport of propranolol, a topical ocular hypotensive β-blocker, in the conjunctival epithelium. The conjunctiva is a thin, mucus secreting, vascularized tissue across which a topically applied drug must pass in order to reach the underlying tissue in the uveal tract via the non-corneal route (12). Although not used clinically for treating glaucoma due to drug tolerance (13), propranolol was chosen as a model drug for probing the role of P-gp in conjunctival β-blocker transport. Evidence for such a role elsewhere includes: (a) propranolol at 12 μM inhibited daunomycin uptake in liver canalicular membrane vesicle by 22% (5); (b) R- and S-propranolol at 125 μM decreased rhodamine123 efflux by P-gp in CEM/VLB100 cells to 43% and 28%, respectively, of the control (14); (c) propranolol increased Vinca alkaloid toxicity in CEM/VLB100 cells as indicated by a 3.5-fold reduction in its IC_{50} (15); and (d) propranolol at 200 μM inhibited 48% of celiprolol secretion in Caco-2 cells (8).

MATERIALS AND METHODS

Materials

[^{3}H] Propranolol (specific activity, 19.3 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). [^{3}H] Metoprolol (specific activity, 1.05 Ci/mmol) and unlabled metoprolol were a gift from Astra Hässle (Mölndal, Sweden). Unlabeled propranolol, alprenolol, atenolol, progesterone, verapamil, rhodamine123, and 2,4-dinitrophenol (2,4-DNP), tetraethylammonium (TEA) bromide, and guanidine were purchased from Sigma Chemical Co. (St. Louis, MO). CsA was obtained from Sandoz Pharmaceuticals (Basel, Switzerland). Cell culture media and supplies were purchased from Gibco (Grand Island, NY), while PC-1 (a serum-free, low-protein, defined medium) culture medium was obtained from BioWhittaker (Walkersville, MD). 4E3 murine monoclonal antibody (mAb) was purchased from Signet (Bedham, MA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was obtained from ICN Pharmaceuticals, Inc. (Aurora, OH). Male Dutch-belted pigmented rabbits, weighing 2.0–2.5 kg, were obtained from American Rabbitry (Los Angeles, CA). Animals were handled in accordance with the Guiding Principles in the Care and Use of Animals (Department of Health, Education, and Welfare, NIH; Publication 80-23).

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Primary Culture of Rabbit Conjunctival Epithelial Cells

Rabbit conjunctival epithelial cells were cultured using a protocol modified from that of Saha *et al.* (16). Briefly, following excision, the conjunctiva was washed in ice-cold Ca^{2+}/Mg^{2+}- free Hanks' balanced salt solution and treated with 0.2% protease (type XIV, 11.2 units/ml) for 60 min at 37°C in 95% air/5% CO_2 to dissociate the cells. The isolated cells were treated with S-MEM supplemented with 10% FBS and 1 mg/ml deoxyribonuclease (DNase I, 2300 Kunitz units/ml) to stop protease reaction, and centrifuged at 100× g for 10 min at room temperature. Resuspended cell pellets in S-MEM were filtered through a 40 μm cell strainer and pelleted again at 100× g for 10 min at room temperature. The final cell pellet was resuspended in Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 (DMEM/F12) medium mixture (1:1) supplemented with 100 U/ml penicillin-streptomycin, 0.5% gentamicin, 0.4% fungizone, 2 mM L-glutamine, 1% ITS^+ (insulin 6.5 μg/ml, transferrin 6.5 μg/ml, selenious acid 6.5 ng/ml, BSA 1.25 mg/ml, and linoleic acid 5.35 mg/ml), 30 μg/ml bovine pituitary extract (BPE), 1 μM hydrocortisone, and 1 ng/ml epidermal growth factor (EGF). These cells were seeded at a density of 1.2×10^6 cells/cm^2 on Transwell inserts (6.5 mm, 0.45 μm pores, Corning-Costar, Cambridge, MA) pre-coated with rat tail collagen I (0.077 mg/ml), and cultured in 5% CO_2 and 95% air at 37°C. From day 2 onward, the growth medium was changed to PC-1 growth medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 0.5% gentamicin and 0.4% fungizone. The transepithelial electrical resistance (TEER) in $k\Omega.cm^2$ and potential difference (PD, apical negative) in mV were estimated with an EVOM epithelial volt-ohmeter (World Precision Instruments, Sarasota, FL). Background TEER and PD contributed by the blank filter and culture medium were corrected for in these measurements. Only those confluent RCEC layers with a resistance above 1 $k\Omega.cm^2$ and a potential difference above 12 mV (usually day 6–8 cells) were used in this study.

Immunohistochemical Detection of P-gp in Cultured RCEC Layers and Excised Conjunctiva

Confluent cell layers on Transwell filters were directly fixed in 4% paraformaldhyde for 20 min at room temperature. One percent bovine serum albumin (BSA) in PBS was used to block non-specific binding sites for 1 hr at room temperature. The cell layers were reacted with primary monoclonal antibody (4E3 mAb at 5 μg/ml) and FITC-conjugated rabbit anti-mouse secondary antibody (0.12 mg/ml) sequentially for 1 hr at room temperature, on either the apical or basolateral side of separate RCEC layers (17). After washing the cells with PBS, the cell layers on the permeable support were cut out and mounted in the coverslip with Vectashield®. Immunofluorescence was observed using confocal microscopy at an excitation wavelength of 488 nm (Zeiss, Germany).

The excised conjunctiva was fixed with 4% paraformaldehyde and infiltrated with 30% sucrose solution. The tissue was snap frozen with Tissue-Tek® O.C.T. Compound in liquid nitrogen and cut into 8 μm sections on cryodisc and collected on slides. After permeabilizing the tissue section with 0.5% Triton X-100 in PBS for 15 min, 1% BSA was used to block non-specific binding sites for 1 hr, all at room temperature. The tissue sections were incubated with primary and secondary antibodies at room temperature using the same method described above. After washing, immunofluorescence was visualized under confocal microscopy (18).

Drug Transport

Prior to each experiment, the confluent cell layers on Transwell inserts were washed and equilibrated with a pH 7.4 bicarbonated Ringer's solution (BRS) containing 1.8 mM $CaCl_2$, 5.6 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM NaH_2PO_4, 25 mM $NaHCO_3$, 116 mM NaCl, 15 mM HEPES, and 5.5 mM D-glucose at 37°C (pH 7.4, 300 mOsm/kg water) for 30 min. Transport study was initiated by adding a dosing solution containing the radiolabeled and unlabeled forms of a given drug (0.3 μCi/ml [^{3}H] propranolol and unlabeled propranolol at varying concentrations; or 0.05 μCi/ml [^{3}H] metoprolol at 0.05 μM or 0.5 μCi/ml [^{3}H] metoprolol at 0.5 μM) to the apical or basolateral compartment. The apical and basolateral volumes were 0.2 and 0.8 ml, respectively. At predetermined times for up to 3 hr, 0.2 ml receiver solution was sampled from the basolateral side (for apical-to-basolateral transport) or 0.1 ml receiver solution from the apical side (for basolateral-to-apical transport), placed in a scintillation vial, and replaced immediately with an equal amount of fresh BRS. The samples was mixed with 5 ml of EconoSafe scintillation cocktail (Research Products International Corp., Mount Prospect, IL) for assay of radioactivity in a liquid scintillation spectrometer (Beckman, Fullerton, CA). The time course of accumulation of radioactivity was analyzed for steady-state flux and, in turn, apparent permeability coefficient, as described subsequently.

Drug Uptake

Prior to each uptake experiment, the cell layers were washed with BRS and preequilibrated for 30 min. Uptake was initiated by spiking the apical fluid with a dosing solution of propranolol ([^{3}H] propranolol (0.3 μCi/ml) in 0.05 μM total propranolol). At 10 min, uptake was terminated by washing the cell layers successively four times with 100 ml ice-cold BRS each. The washed cell layers were solubilized in 0.5 ml of 0.5% Triton X-100 solution. Twenty μl of the cell lysate was taken for protein assay using the method of Bradford (19), with bovine serum albumin as a standard. The rest of the cell lysate was mixed with 5 ml of EconoSafe scintillation fluid for measurement of radioactivity. Drug uptake was expressed as amount of drug accumulated per mg of cellular protein over the duration of measurement.

Propranolol (0.05 μM) uptake in the presence of 100 μM P-gp inhibitors (CsA, progesterone, rhodamine123 and verapamil; all dissolved in 0.1% dimethyl sulfoxide (DMSO)) was carried out in essentially the same manner. In the 4E3 mAb experiment, the cell layers were pre-incubated with 5 μg/ml 4E3 mAb in the apical bathing fluid only. In the 2,4-DNP experiment, 0.2 mM 2,4-DNP was added to both apical and basolateral bathing fluids in glucose-free BRS for 1 hr before dosing. In those experiments involving β-blockers (hydrophilic atenolol, moderately lipophilic metoprolol, and highly lipophilic alprenolol) or organic cation transporter substrates (TEA and guanidine), 100 μM of each compound was present in the final donor solution.

Data Analysis

The steady-state flux was estimated from the slope of the linear portion of a plot of a cumulative amount of drug appearing in the receiver fluid as a function of time. The apparent permeability coefficient (Papp) of unidirectional fluxes for solutes was estimated by normalizing the flux, dQ/dt (mol/sec), against the nominal surface area ($A = 0.33\ cm^2$) and initial solute concentration in the donor fluid, C_0 (mol/ml), or $Papp = (dQ/dt)/(A \times C_0)$. The kinetic parameters for propranolol transport in cultured RCEC were estimated by fitting the flux against donor propranolol concentration using a nonlinear least-square curve fitting program (Table Curve 2D, Jandel Scientific Inc., San Rafael, CA). The assumption is that propranolol transport is comprised of two components: influx via simple diffusion and efflux (that is saturable) via P-gp:

$$J_{ab} = K_d[C] - J_{max}[C]/(K_m + [C])$$

$$J_{ba} = K_d[C] + J_{max}[C]/(K_m + [C])$$

$$J_{net} = J_{ba} - J_{ab} = 2\ J_{max}[C]/(K_m + [C])$$

where J_{ab} is the unidirectional flux in the apical-to-basolateral direction and J_{ba} is that in the opposite direction, J_{net} is the net propranolol flux in the basolateral-to-apical direction, K_d is the non-saturable (i.e. passive diffusional) permeation rate, J_{max} is the maximum saturable flux, K_m is the corresponding Michaelis-Menten constant, and [C] is the initial donor propranolol concentration.

Two-tailed Student's t-test for unpaired data or one-way analysis of variance was used to determine the significance of difference between means of more than two data groups. Post-hoc comparisons were made using Tukey's multiple comparison test to contrast statistical significance among group (≥ 3) means. $P < 0.05$ was considered as statistically significant.

RESULTS

Localization of P-gp

Apical (Fig. 1A), but not basolateral (Fig. 1C), aspect of the cultured conjunctival epithelial cell layers observed showed positive staining of cells in the presence of 4E3 mAb under confocal laser-scanning microscopy. The absence of any staining in the cell layers with FITC-conjugated secondary antibody incubation alone (i.e., without 4E3 mAb pre-incubation) served as a negative control (Fig. 1B).

Immunohistochemistry of P-gp in frozen tissue sections of rabbit conjunctiva showed positive staining of the outermost cell layer of the epithelium, weak staining in cytoplasm of the other portions of the cell layers, and no staining in the endothelium (Fig. 2A). Fig. 2B is the corresponding phase-contrast photomicrograph. The absence of any specific staining in the tissue sections incubated with FITC-conjugated secondary antibody alone (i.e., without 4E3 mAb pre-incubation) served as a negative control (Fig. 2C). Fig. 2D shows the corresponding phase-contrast photomicrograph.

Conjunctival Propranolol and Metoprolol Transport

The unidirectional fluxes of propranolol at varying concentrations in either the apical-to-basolateral or basolateral-to-apical direction across cultured RCEC were shown in Fig. 3. Basolateral-to-apical fluxes were significantly greater than apical-to-basolateral fluxes at all concentrations except 0.5 μM (The diffusion component may be predominant at this concentration). The ratios of fluxes estimated from the basolateral-to-apical transport over that from the apical-to-basolateral transport were 1.9 (0.0125 μM), 2.2 (0.025 μM), 2.4 (0.05 μM), 1.8 (0.1 μM), and 1.4 (0.2 μM). The net flux, occurring in the basolateral-to-apical direction, is in concordance with the presence of a saturable kinetic process with a K_m of 71.5 ± 24.0 nM and J_{max} of 1.45 ± 0.17 pmol/cm^2/hr (Fig. 3). By contrast, metoprolol transport at 0.05 and 0.5 μM across RCEC layers showed no directional preference (Table I).

Apical Propranolol Uptake in Cultured Conjunctival Epithelial Cell Layers

As shown in Fig. 4, apical uptake of propranolol at 50 nM was significantly increased ($p < 0.05$) in the presence of P-gp substrates, all at 100 μM: CsA (66%), progesterone (43%), rhodamine123 (49%), and verapamil (52%). Apical presence

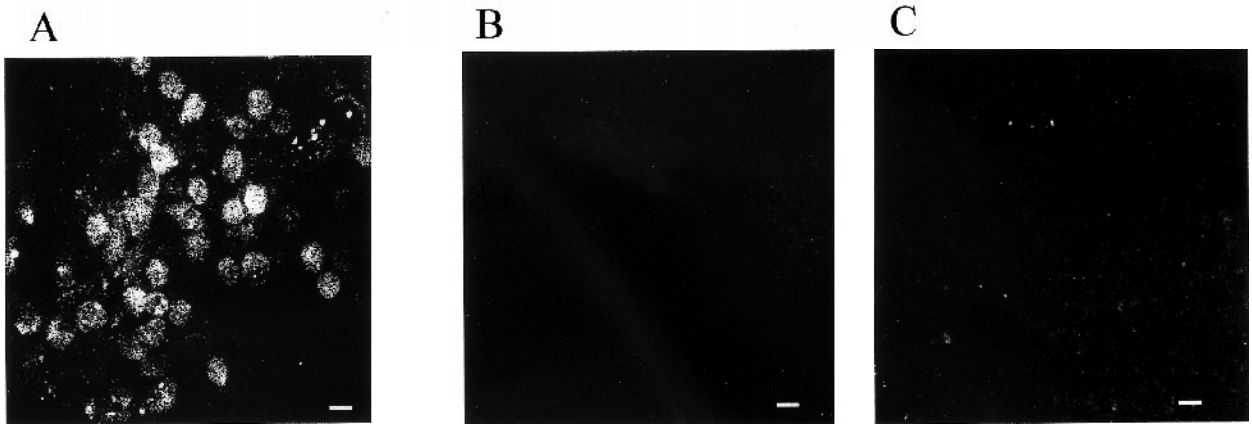

Fig. 1. Immunolocalization of P-glycoprotein in cultured RCEC layers by confocal laser-scanning microscopy. Panels A and B are the immunofluorescence images observed with and without 4E3 mAb treatment on the apical side, respectively. Panel C is the immunofluorescence picture found with 4E3 mAb added to the basolateral fluid (original magnification 780×). Bar lengths are 10 μm.

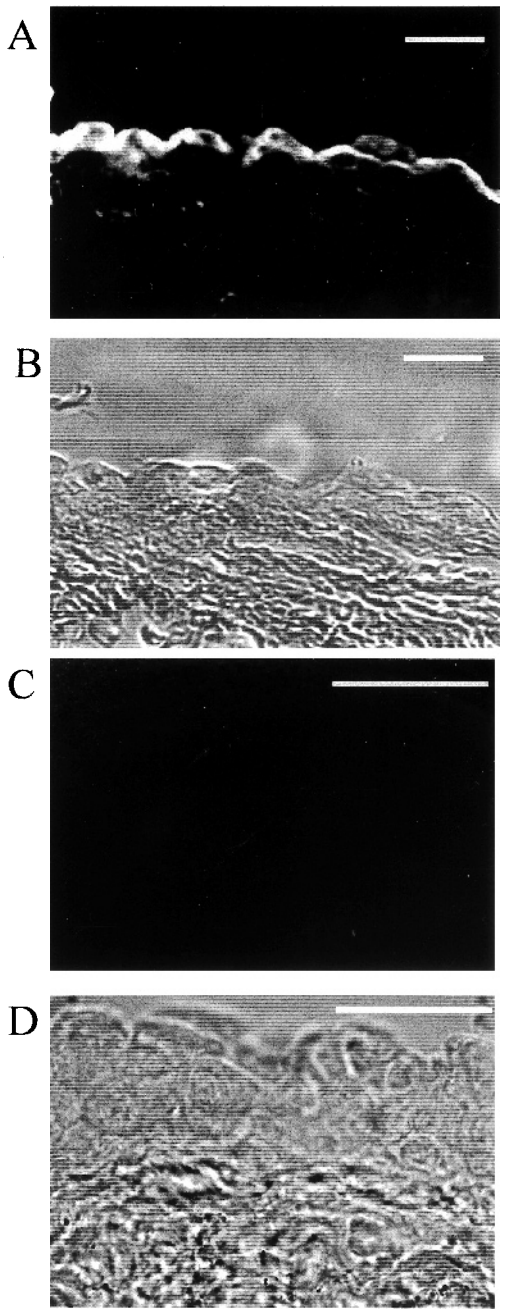

Fig. 2. Immunohistochemistry of P-glycoprotein on frozen tissue sections of excised rabbit conjunctiva. Panel A: immunofluorescence with 4E3 mAb treatment, panel B: corresponding image obtained with phase contrast (original magnification 2200×); panel C: immunofluorescence without 4E3 mAb treatment, and panel D: corresponding image acquired with phase contrast (original magnification 4400x). Bar lengths are 10 μm.

of 4E3 mAb at 5 μg/ml and apical and basolateral presence of 2,4-DNP at 0.2 mM increased the uptake of 50 nM apical propranolol concentration by 55% and 53%, respectively. By contrast, neither the β-blockers (atenolol, metoprolol, and alprenolol) nor the organic cation transporter substrates (TEA and guanidine), all at 100 μM, affected apical 50 nM propranolol uptake (Fig. 4). Lowering the temperature to 4°C reduced apical propranolol uptake by 76%.

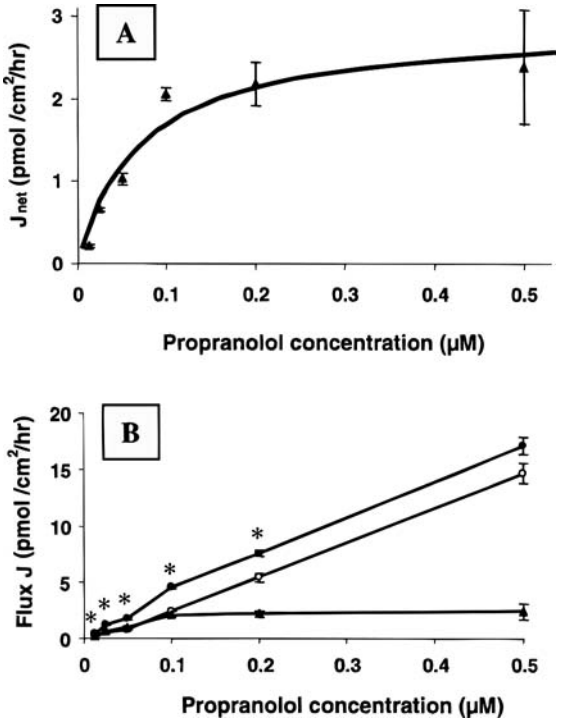

Fig. 3. Panel **A**: Net flux of propranolol across cultured rabbit conjunctival epithelial cell layers as a function of propranolol concentration. Panel **B**: Unidirectional fluxes and net flux of propranolol at 0.0125 to 0.5 μM. The data are mean ± s.e.m. (n = 4–6) (* $p < 0.05$). Key: ▲, Net secretory flux ($J_{net} = J_{ba} - J_{ab}$); ○, J_{ab} (flux in the apical-to-basolateral direction); ●, J_{ba} (flux in the basolateral-to-apical direction).

DISCUSSION

This study provides immunohistochemical evidence for the apical localization of a P-gp efflux pump in cultured RCEC layers (Fig. 1) and excised conjunctival epithelial tissue (Fig. 2). Probably due to the P-gp drug efflux pump, the transport of propranolol across the cultured cell layers was asymmetric, favoring secretion which was saturable (Fig. 3). The observed K_m for conjunctival propranolol efflux is 71.5 nM. This is similar to the K_m of calcein-acetoxymethylester (50 nM) (20) and rhodamine123 (34 nM) (14), but is much smaller than that for idarubicin (1 μM) (21). At low propranolol concentrations (0.0125–0.5 μM), the contribution of active transport to the

Table I. Apparent Permeability Coefficients (Papp) of Propranolol and Metoprolol Across Cultured Rabbit Conjunctival Epithelial Cells

Drug	Log P[a]	Papp ($\times 10^{-5}$ cm/s) ab[b]	Papp ($\times 10^{-5}$ cm/s) ba[c]
Propranolol (0.05 μM)	3.21	0.42 ± 0.02	1.00 ± 0.07*
Metoprolol (0.05 μM)	1.88	1.23 ± 0.01	1.31 ± 0.04
Metoprolol (0.5 μM)	1.88	1.31 ± 0.07	1.41 ± 0.04

Note: Data represent mean ± s.e.m. (n = 4–6).
[a] From ref. 28.
[b] Apical-to-basolateral direction.
[c] Basolateral-to-apical direction.
* Significantly different from that in ab direction ($p < 0.05$).

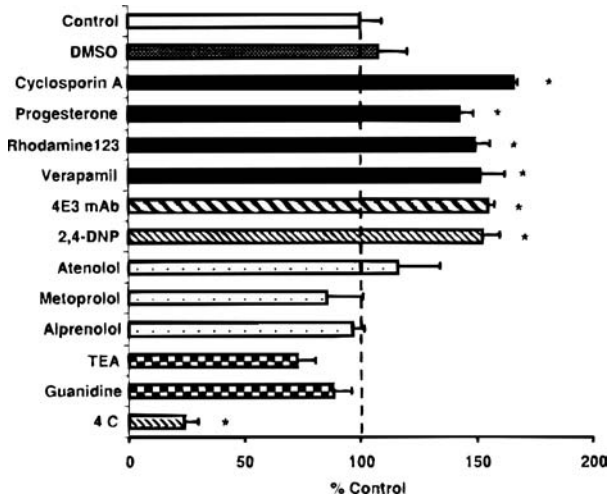

Fig. 4. Ten min uptake of propranolol at a donor concentration of 0.05 μM in the apical fluid in the presence of competing substrates (CsA, progesterone, rhodamine123 and verapamil), β-blockers (atenolol, metoprolol, and alprenolol) and organic cation transporter substrates (TEA and guanidine), all at 100 μM, except for 5 μg/ml 4E3 mAb and 0.2 mM 2,4-DNP. The data are mean ± s.e.m. (n = 4–6) (*$p < 0.05$).

overall flux in the basolateral-to-apical direction ranges from 58% to 14%. Since we did observe apical localization of P-gp in the conjunctival epithelial cells (Figs. 1 and 2), uptake over a short period from the apical side may be more appropriate for determining P-gp function than the prolonged flux studies that may be fraught with non-specific effects caused by these inhibitors/modulators.

Apical propranolol uptake in cultured conjunctival epithelial cell layers was increased by 43% to 66% in the presence of P-gp substrates and modulators—CsA, progesterone, rhodamine123, and verapamil, further suggesting the involvement of P-gp in restricting apical conjunctival propranolol uptake. The uncoupling agent (2,4-DNP) increased apical uptake of propranolol by 53%, suggesting that by blocking mitochondrial ATP synthesis, the cellular energy was depleted to lead to a decrease in P-gp pump activity. 4E3 mAb, which recognizes the external portion of P-gp, increased propranolol uptake by 55%, indicating that antibody binding decreased P-gp activity (Fig. 4). This finding also suggests that this antibody might be useful in the attenuation of multidrug resistance involvement or targeting drug to the specific tissue that bears P-gp. For example, using an immunotoxin composed of MRK16 (a P-gp antibody) coupled to *Pseudomonas* exotoxin (MRK16-PE), multidrug-resistant cells were destroyed, whereas cells not expressing the P-gp were not affected (22).

It is of interest to know whether or not the transport of other β-blockers is similarly affected by P-gp on the basis of competitive inhibition of propranolol uptake. Not only hydrophilic atenolol (log P = 0.16) but also moderately lipophilic metoprolol (log P = 1.88) and highly lipophilic alprenolol (log P = 3.1) failed to affect propranolol uptake (Fig. 4). In addition, metoprolol transport across cultured RCEC did not show directional dependence (Table I), strongly suggesting that this particular β-blocker may not be a P-gp substrate. Nevertheless, Karlsson *et al.* (8) did find that, besides propranolol, atenolol and metoprolol also inhibited basolateral-to-apical secretion of celiprolol in Caco-2 cells. Two distinct transport systems (i.e., P-gp and organic cation-H^+ exchanger) were tested. Although inhibition of P-gp by atenolol and metoprolol could not be completely ruled out by these investigators, the inhibition effect on celiprolol by β-blockers may be due to organic cation-H^+ exchanger.

Of the β-blockers evaluated, only propranolol has a naphthalene ring. Although P-gp substrates encompass a wide range of chemical structures and different classes of drugs (15,23), including detergents, antibiotics, antimalarials, antihypertensives, and immunosuppressives, a typical P-gp substrate is likely to be hydrophobic with two planar aromatic rings as well as a tertiary nitrogen that confers a positive charge at physiological pH (23). To date, the P-gp "pharmacophore" has not been well defined and remains a topic to be explored further.

At 4°C, the activity of P-gp efflux pump system should be low and therefore the uptake of propranolol by conjunctival epithelial cells would be expected to be increased. That the uptake of propranolol at 4°C was only 24% of that at 37°C suggests a non-passive component in its uptake that, in turn, serves to rate-limit its efflux mediated by P-gp. Indeed, Kurihara *et al.* (24) reported that propranolol transported into the brain microvessel endothelial cells occurred via facilitated diffusion. The organic cation transporter (OCT), which has been demonstrated in the rabbit conjunctiva (25), did not appear to be involved. This is because neither TEA nor guanidine, which are known substrates of OCT (26), affected propranolol uptake in conjunctival epithelial cells (Fig. 4).

Besides decreased diffusion at 4°C, another possible reason for the uptake reduction is decresed propranolol binding. In fact, Kawazu *et al.* (10) observed significantly lower uptake of CsA in cultured corneal epithelial cells at 4°C and 25°C than that at 37°C. They concluded CsA uptake was energy dependent and postulated the presence of a depot for CsA binding in the form of cyclophilin. Kurihara *et al.* (24) also suggested the existence of a propranolol binding or trapping compartment in bovine brain endothelial cells. In addition, due to the high lipophilicity, propranolol may easily bind to membrane as well as receptors such as β-adrenergic receptor (27). At 4°C, recycling of membrane and receptors shuts down, thereby decreasing propranolol uptake. In short, the reduction of propranolol uptake at 4°C may be contributed by reduction of passive diffusion, binding ability, facilitated diffusion (if there is), and membrane-associated uptake. Further work is required to characterize carrier-mediated conjunctival propranolol uptake.

In summary, the energy-dependent efflux pump P-gp appears to be predominantly located on the apical plasma membrane of the conjunctival epithelium. It may play an important role in restricting the conjunctival absorption of some lipophilic drugs.

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REFERENCES

1. W. T. Bellamy. P-glycoprotein and multidrug resistance. *Annu. Rev. Pharmacol. Toxicol.* **36**:161–183 (1996).
2. K. Hosoya, K. J. Kim, and V. H. L. Lee. Age-dependent expression

of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm. Res.* **13**:885–890 (1996).
3. P. Saha, J. J. Yang, and V. H. L. Lee. Existence of a P-glycoprotein drug efflux pump in cultured rabbit conjunctival epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **39**:1221–1226 (1998).
4. T. Terao, E. Hisanaga, Y. Sai, I. Tamai, and A. Tsuji. Active secretion of drugs from the small intestinal epithelium in rats by P-glycoprotein functioning as an absorption barrier. *J. Pharm. Pharmacol.* **48**:1083–1089 (1996).
5. Y. Kwon, A. V. Kamath, and M. E. Morris. Inhibitors of P-glycoprotein-mediated daunomycin transport in rat liver canalicular membrane vesicles. *J. Pharm. Sci.* **85**:935–939 (1996).
6. B. Leu and J. Huang. Inhibition of intestinal P-glycoprotein and effects on etoposide absorption. *Cancer Chemother. Pharmacol.* **35**:432–436 (1995).
7. A. J. Dudley and C. D. A. Brown. Mediation of cimetidine secretion by P-glycoprotein and a novel H^+-coupled mechanism in cultured renal epithelial monolayers of LLC-PK1 cells. *Br. J. Pharmacol.* **117**:1139–1144 (1996).
8. J. Karlsson, S.-M. Kuo, J. Ziemniak, and P. Artusson. Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein. *Br. J. Pharmacol.* **110**:1009–1016 (1993).
9. F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan, and M. C. Willingham. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. USA* **84**:7735–7738 (1987).
10. K. Kawazu, K. Yamada, M. Nakamura, and A. Ota. Characterization of cyclosporin A transport in cultured rabbit corneal epithelial cells: P-glycoprotein transport activity and binding to cyclophilin. *Invest. Ophthalmol. Vis. Sci.* **40**: 1738–1744 (1999).
11. J. A. Holash and P. A. Stewart. The relationship of astrocyte-like cells to the vessels that contribute to the blood-ocular barriers. *Brain Res.* **629**:218–224 (1993).
12. U. B. Kompella, K. J. Kim, and V. H. L. Lee. Active chloride transport in the pigmented rabbit conjunctiva. *Curr. Eye Res.* **12**:1041–1048 (1993).
13. L. Bonomi, S. Perfetti, E. Noya, R. Bellucci, and F. Massa. Comparison of the effects of nine beta-adrenergic blocking agents on intraocular pressure in rabbits. *Albrecht v. Graefes Arch. Klin. Exp. Ophthal.* **210**:1–8 (1979).
14. P. W. Wigler. Cellular drug efflux and reversal therapy of cancer. *J. Bioenerg. Biomembr.* **28**:279–284 (1996).
15. J. M. Zamora, H. L. Pearce, and W. T. Beck. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol. Pharmacol.* **33**:454–462 (1988).
16. P. Saha, K. J. Kim, and V. H. L. Lee. A primary culture model of rabbit conjunctival epithelial cells exhibiting tight barrier properties. *Curr. Eye Res.* **15**:1163–1169 (1996).
17. R. J. Arceci, K. Stieglitz, J. Bras, A. Schinkel, F. Bass, and J. Croop. Monoclonal antibody to an external epitope of the human mdr1 P-glycoprotein. *Cancer Res.* **15**:310–317 (1993).
18. R. Evers, G. J. Zaman, L. van Deemter, H. Jansen, J. Calafat, L. C. Oomen, R. P. Oude Elferink, P. Borst, and A. H. Schinkel. Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *J. Clin. Invest.* **97**:1211–1218 (1996).
19. M. M. Bradford. A rapid and sensetive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
20. M. Essodaigui, H. J. Broxterman, and A. Garnier-Suillerot. Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. *Biochem.* **37**:2243–2250 (1998).
21. S. Mankhetkorn, F. Dubru, J. Hesschenbrouck, M. Fiallo, and A. Garnier-Suillerot. Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(S)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 cells. *Mol. Pharmacol.* **49**:532–539 (1996).
22. D. J. Fitzgerald, M. C. Willingham, C. O. Cardarelli, H. Hamada, T. Tsuruo, M. M. Gottesman, and I. Pastan. A monoclonal antibody-Pseudomonas toxin conjugate that specifically kills multidrug-resistant cells. *Proc. Natl. Acad. Sci. USA* **84**:4288–4292 (1987).
23. W. T. Beck. Modulators of P-glycoprotein-associated multidrug resistance. *Cancer Treat. Res.* **57**:151–170 (1991).
24. A. Kurihara, H. Suzuki, Y. Sawada, Y. Sugiyama, T. Iga, and M. Hanano. Uptake of propranolol by microvessels isolated from bovine brain. *J. Pharm. Sci.* **76**:759–764 (1987).
25. H. Ueda, Y. Horibe, K. J. Kim, and V. H. L. Lee. Functional characterization of organic cation drug transport in the pigmented rabbit conjunctiva. *Invest. Ophthalmol. Vis. Sci.* In press.
26. Y. Urakami, M. Okuda, S. Masuda, H. Saito, and K. I. Inui. Functional characteristics and membrane localization of rat multispecific organic cation transporters, OCT1 and OCT2, mediating tubular secretion of cationic drugs. *J. Pharmacol. Exp. Ther.* **287**:800–805 (1998).
27. S. Marullo, V. Faundez, and R. B. Kelly. Beta 2-adrenergic receptor endocytic pathway is controlled by a saturable mechanism distinct from that of transferrin receptor. *Receptors Channels* **6**:255–269 (1999).
28. C. Hansch, A. Leo, and D. Hoekman. Exploring QSAR: Hydrophobic, electronic, and steric constants. ACS Professional Ref. Book. Washington DC, 1995.