Functional Characterization of Organic Cation Drug Transport in the Pigmented Rabbit Conjunctiva

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PURPOSE. To characterize carrier-mediated organic cation drug transport in the rabbit conjunctiva.

METHODS. The transport of [14C]guanidine, the model substrate, in the excised pigmented rabbit conjunctiva was evaluated in the modified Ussing chamber. Tetraethylammonium (TEA) transport also was investigated to determine substrate specificity.

RESULTS. The apparent permeability coefficient for guanidine and TEA in the mucosal-to-serosal (ms) direction was 5.4 and 49.6 times greater than that in the scrostral-to-mucosal (sm) direction, respectively. Guanidine transport in the ms (but not sm) direction revealed temperature and concentration dependency over 0.02 to 10 mM with an apparent Michaelis-Menten constant of 3.1 mM and a maximal flux of 11.4 nmol/(cm² h). Net guanidine transport measured at 0.1 mM across the conjunctiva was decreased by 71% or 82%, respectively, on the addition of 1 μM valinomycin (a K⁺ ionophore) in both bathing fluids or in a high K⁺ buffer in the mucosal fluid. Interestingly, net guanidine transport was reduced, rather than enhanced, by 63% upon acidifying the mucosal bathing fluid. By contrast, net guanidine transport was not affected by the serosal presence of 0.5 mM ouabain (a Na⁺,K⁺-ATPase inhibitor), by the mucosal and serosal presence of 0.1 μM monensin (a Na⁺ ionophore) or 0.3 μM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP, a H⁺ ionophore). Guanidine transport in the ms direction was polyspecific, as indicated by the 48% to 82% inhibition by structurally diverse amines. In particular, guanidine ms transport was inhibited by the antiglaucoma drugs dipivefrine (72%), brimonidine (70%), and carbachol (78%).

CONCLUSIONS. A carrier-mediated organic cation transport process appears to exist in the conjunctiva, mediating the absorption of organic amines, including certain amine-type ophthalmic drugs. This process may be driven by an inside-negative apical membrane potential difference. (Invest Ophthalmol Vis Sci. 2000;41:870–876)

Many endogenous amines (e.g., epinephrine, choline, dopamine, and guanidine) as well as a number of xenobiotics exist as cations at physiological pH. These compounds are known to be transported in the intestinal, hepatic, renal, alveolar, and choroid plexus epithelia, as the guanidinium ion at physiological pH, was chosen as a model substrate. It has been used to characterize OC transport processes in the ocular tissues have not been systematically studied to date. Conceivably, such processes may exist in the conjunctival (and corneal) epithelial cells to reabsorb various endogenous amines such as epinephrine, dopamine, histamine, and serotonin in the tear fluid. These same transport processes may also facilitate, at least in part, the absorption of topicaly applied ophthalmic drugs that are positively charged at physiological pH, such as carbachol, phystostigmine, pilocarpine, dipivefrine, apraclonidine, and brimonidine.

The present study represents our ongoing effort to characterize active drug transport processes in the conjunctiva. We undertook the present study to characterize OC transport processes in the rabbit conjunctiva with respect to directionality, temperature dependency, saturability, substrate specificity, and driving force. [14C]Guanidine (pKa = 12.5), a primary amine that exists almost exclusively as the guanidinium ion at physiological pH, was chosen as a model substrate. It has been used to characterize OC transport processes in the rabbit lung, human placenta and kidney, and human choriocarcinoma cell line (JAR).
\[^{14}\text{C}\]\text{Tetraethylammonium (TEA)} was also used as an additional substrate.\(^{11,12}\)

**Materials and Methods**

**Animals**

Male Dutch-belted pigmented rabbits, weighing 2.5 to 3.0 kg, were purchased from Irish Farms (Los Angeles, CA). The investigations using rabbits described in this report conformed to the Guiding Principles in the Care and Use of Animals (DHENV Publication, NIH 80-23) and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**Chemicals**

\[^{14}\text{C}\]\text{Guanidine (55 mCi/mmol)} was purchased from Moravek Biochemicals (Brea, CA). \[^{12}\text{C}\]\text{TEA (55 mCi/mmol)} was purchased from American Radiolabeled Chemicals (St. Louis, MO). \[^{1}\text{H}\]\text{arginine HCl (58 Ci:mmol)} was obtained from Amer sham Co. (Downers Grove, IL). Guanidine, TEA, choline, amiloride, procainamide, clonidine, L-arginine, D-arginine, carbachol, brimonidine, ouabain, valinomycin, monensin, and carbonyl cyanide \(p\)-(trifluoromethoxy) phenyl-hydrazone (FCCP) were obtained from Sigma Chemical Co. (St. Louis, MO). Dipivefrine hydrochloride was kindly provided by Santen Pharmaceutical Co. (Osaka, Japan).

**Solutions**

Unless otherwise indicated, all experiments were conducted in the bicarbonated Ringer’s solution maintained at 37°C and pH 7.4 under 95% air/5% CO\(_2\). The bicarbonated Ringer’s solution contained 111.5 mM NaCl, 4.8 mM KCl, 0.75 mM NaH\(_2\)PO\(_4\), 29.2 mM NaHCO\(_3\), 1.04 mM CaCl\(_2\), 0.74 mM MgCl\(_2\), and 5 mM D-glucose. Bicarbonated Ringer’s solutions of different pHS were prepared by adding 15 mM 2-[\(\text{N}-\text{morpholino}\)]ethanesulfonic acid (MES, at pH 5.0 or 6.0) or N-[\(\text{2-hydroxyethyl}\)]pipеразине-\(\text{N}-\text{2-ethanesulfonic acid} (HEPES, at pH 8.0) with the normal bicarbonated Ringer’s solution and titrated with either HCl or NaOH to the respective pH. The osmolality of all buffers was adjusted to 300 mOsm/kg H\(_2\)O by adding mannitol, if needed.

**Tissue Preparation**

We have previously reported the detailed procedure for preparing the excised rabbit conjunctiva for transport studies in the modified Ussing chamber.\(^{28}\) Briefly, rabbits were euthanized with an injection of 85 mg/kg sodium pentobarbital solution into a marginal ear vein, and the entire eye ball was removed from the orbit. After trimming, the excised conjunctiva was mounted in the tissue adapter with a circular aperture of 1.0 cm\(^2\), which was then placed in a modified Ussing chamber. The bathing solutions (6 ml each) were bubbled with 95% air/5% CO\(_2\) to maintain the pH at 7.4 and to provide adequate agitation. The Ussing chamber assembly was maintained at 36 ± 1°C with a circulating water bath.

**Bioelectric Parameter Measurements**

All experiments were performed under short-circuit condition with the use of an automatic voltage-clamp device (558C-5; Bioengineering Department, University of Iowa, Iowa City, IA). Potential difference (PD) was measured with two matched calomel electrodes. Two polyethylene (PE 90) bridges (containing 4% agar in 3 M KCl), whose tips were located near the center of tissue surfaces, were used to electrically connect the reservoir fluid to electrode wells. The electrical output of calomel electrodes was amplified by the voltage-clamp unit. Direct current flowing across the tissue was sent with a pair of matched Ag/AgCl electrodes with conducting agar bridges, whose tips were positioned away from tissue surfaces at the far ends of two reservoirs. The short-circuit current (\(I_{sc}\)) flowing in the bath-tissue-bath circuit was monitored and recorded on a strip chart recorder (Kipp and Zonen, Delft, The Netherlands). At 60-second intervals, a 2-mV pulse (\(\Delta V\)) was imposed for 3 seconds across the short-circuited tissue to estimate the transepithelial electrical resistance (TEER) as a surface area normalized ratio of applied voltage pulse to the observed deflection in resultant current (\(\Delta I\)) flowing on top of \(I_{sc}\) (\(\Delta I_{sc}\)).

**Measurements of Guanidine and TEA Fluxes**

Guanidine flux measurement was initiated by adding \[^{14}\text{C}\]\text{guanidine (1 \(\mu\)Ci/ml) and/or a varying amount of unlabeled guanidine to the mucosal or serosal donor fluid, after the tissue was equilibrated, as indicated by a stable \(I_{sc}\). At 0.5, 1, 1.5, 2, and 3 hours, a 0.5-ml aliquot was collected from the receiver fluid for assay of radioactivity in a liquid scintillation counter (Beckman, Fullerton, CA). The aliquot removed was immediately replaced with an equal volume of fresh buffer. For the TEA flux measurement, \[^{14}\text{C}\]\text{TEA (1 \(\mu\)Ci/ml) was used. To determine the driving force for OCT substrate transport, the conjunctiva was preincubated with a pharmacological agent for 60 minutes before the addition of \[^{14}\text{C}\]\text{guanidine, except for ouabain, which was preincubated for 90 minutes.\(^{21,29}\) To determine substrate specificity, the compound of interest (unlabeled) was added to the donor fluid concurrently with \[^{14}\text{C}\]\text{guanidine.}

**Data Analysis**

Area normalized permeation amount (\(Q, \text{mol/cm}^2\)) for guanidine and TEA was calculated from Equation 1, where

\[
Q = [\text{total cpm in receiver fluid} \times \text{specific activity (mol/cpm)}/[\text{area (cm}^2)]\]  

Normalized unidirectional fluxes (\(J, \text{mol/cm}^2 \times \text{h}\)) were then estimated from the steady state (60–180 min) slope of a plot of cumulative amount of penetrant appearing in the receiver fluid versus time. The apparent permeability coefficient (\(P_{app}\)) was calculated by further normalizing the flux against the initial substrate concentration in the donor fluid (mol/cm\(^2\)). The kinetic parameters for guanidine transport across the conjunctiva were estimated by fitting the observed guanidine flux data to Equation 2 using MULTI,\(^{30}\) a software program for nonlinear least-square regression analysis:

\[
J = J_{\text{max}}[C]/(K_m + [C]) + K_\text{dl}C 
\]
where \( [C] \) is substrate concentration, \( J_{\text{max}} \) is maximal flux, \( K_m \) is apparent Michaelis–Menten constant, and \( K_d \) is the nonsaturable (i.e., diffusional) permeation rate. Unpaired, two-tailed Student’s \( t \)-test was used to determine a statistical difference between two group means. When comparing three or more group means, one-way analysis of variance (ANOVA) was used. Statistical significance among the group means was determined by the modified Fisher’s least-squared difference approach. A \( P < 0.05 \) was considered significant.

**RESULTS**

**Directionality of Guanidine and TEA Transport**

As shown in Figure 1, the transport profile of guanidine and TEA in the mucosal-to-serosal (ms) and serosal-to-mucosal (sm) directions at pH 7.4 and 37°C showed typical pseudo steady state characteristics after a lag time of 30 to 40 minutes. The \( P_{\text{app}} \) of guanidine in the ms and sm directions were \( 4.38 \pm 0.27 \times 10^{-6} \) cm/s and \( 0.81 \pm 0.03 \times 10^{-6} \) cm/s, respectively. The corresponding values for TEA were \( 8.92 \pm 1.73 \times 10^{-6} \) cm/s and \( 0.18 \pm 0.04 \times 10^{-6} \) cm/s. The ms fluxes were, therefore, 5.4- and 49.6-fold higher than sm fluxes for guanidine and TEA transport, respectively.

**Concentration and Temperature Dependency of Guanidine Transport**

Guanidine fluxes in the ms direction showed saturability over 0.02 to 10 mM (Fig. 2). The corresponding \( K_m, J_{\text{max}} \) and \( K_d \) were estimated to be \( 3.1 \pm 0.5 \) mM, \( 11.4 \pm 1.6 \) nmol/(cm\(^2\)·h), and \( 0.47 \pm 0.06 \times 10^{-6} \) cm/s, respectively. By contrast, a linear relationship between flux and concentration of guanidine was observed at 4°C. The corresponding slope was \( 0.43 \pm 0.04 \times 10^{-6} \) cm/s, which was not significantly different from the \( K_d \) value at 37°C \((P > 0.05)\). No \( I_{sc} \) or TEER changes were observed at any of the concentrations studied.

**pH Dependency of Guanidine Transport**

As shown in Figure 3, when the mucosal side of the conjunctiva was exposed to an acidic buffer (pH 5.0 and 6.0), the net guanidine \( P_{\text{app}} \) of guanidine in the ms and sm directions were \( 4.38 \pm 0.27 \times 10^{-6} \) cm/s and \( 0.81 \pm 0.03 \times 10^{-6} \) cm/s, respectively. The corresponding values for TEA were \( 8.92 \pm 1.73 \times 10^{-6} \) cm/s and \( 0.18 \pm 0.04 \times 10^{-6} \) cm/s. The ms fluxes were, therefore, 5.4- and 49.6-fold higher than sm fluxes for guanidine and TEA transport, respectively.

**Guanidine Transport in the Presence of Ionophores**

Guanidine transport in the ms and sm directions was studied in the presence of various ionophores \([1 \mu M \text{valinomycin (K}^+ \text{ionophore}), 0.1 \mu M \text{monensin (Na}^+ \text{ionophore}), \) and \( 0.3 \mu M \text{FCCP (H}^+ \text{ionophore)}) in both bathing fluids. At these concentrations, there were no significant changes in the TEER, although decreases in \( I_{sc} \) by valinomycin (24%), monensin (65%),

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**FIGURE 1.** Time courses of \([^{14}C]\text{TEA (a) and } [^{14}C]\text{guanidine (b) transport across the pigmented rabbit conjunctiva in the mucosal-to-serosal (ms) and serosal-to-mucosal (sm) directions. All experiments were conducted in the presence of } [^{14}C]\text{guanidine or } [^{14}C]\text{TEA at 1 } \mu \text{Ci/ml (18 } \mu \text{M). Data points represent mean } \pm \text{ SEM (} n = 3–6). \) Where not visible, the error bar is smaller than the size of the symbol. ( ), ms direction; ( ), sm direction.

**FIGURE 2.** Total mucosal-to-serosal guanidine fluxes in the pigmented rabbit conjunctiva as a function of guanidine concentration. All experiments were conducted in the presence of 1 \( \mu \text{Ci/ml (18 } \mu \text{M) } [^{14}C]\text{guanidine and } 0.02 \text{ to } 10 \text{ mM unlabeled guanidine. Data points represent mean } \pm \text{ SEM (} n = 3–6). \) ( ), total flux at 37°C; ( ), total flux at 4°C.
and FCCP (55%) were evident (data not shown). Ouabain at 0.5 mM also abolished the $I_{sc}$, as reported previously (data not shown).28 As shown in Table 1, the guanidine $P_{app}$ in the sm direction was not affected by any of the pharmacological agent tested ($P > 0.05$). However, net guanidine transport was significantly decreased by 71% and 82% ($P < 0.05$) by 1 mM valinomycin when present, respectively, in both bathing fluids and in a high K+ (116.3 mM) buffer in the mucosal fluid, suggesting the involvement of an inside negative cell membrane potential-dependent transport process. By contrast, net guanidine transport was not affected by 0.5 mM ouabain, 0.1 mM monensin, or 0.3 mM FCCP.

**Substrate Specificity**

The effect of various OC compounds added mucosally at 1 mM on guanidine $P_{app}$ in the ms direction is shown in Figure 4. (For some competing compounds, 0.1 mM was used instead to avoid a possible drug effect on tight junctional integrity.) These compounds include unlabeled guanidine, a primary amine (amiloride), a secondary amine (dipivefrine), a tertiary amine (propanamide), quaternary amines (TEA, choline, and carbachol), heterocyclic amines (clonidine and brimonidine), and basic amino acids (D-arginine and L-arginine). Guanidine $P_{app}$ was significantly inhibited by all the OC compounds tested ($P < 0.05$), notably by amiloride, TEA, and choline (>80%). Interestingly, the guanidine $P_{app}$ in the ms direction was decreased by the antiglaucoma drugs dipivefrine (72%), brimonidine (70%), and carbachol (78%). In the case of TEA, its $P_{app}$ was significantly inhibited by unlabeled TEA and guanidine at 1 mM by 94% and 47%, respectively (Table 2).

**DISCUSSION**

**Kinetics of the Conjunctival Guanidine Transport Process**

We have obtained evidence for the mucosal presence of a carrier-mediated OC transport process in the conjunctiva that appears to work in concert with passive diffusion to mediate the overall transport of organic cations in the ms direction. Passive diffusion contributes 32% to overall guanidine transport at 0.1 mM, 38% at 1 mM, and 66% at 10 mM (Fig. 2). Given that the $K_{g}$ value (0.47 ± 0.06 × 10^{-6} cm/s) in Figure 2 is comparable to 0.55 × 10^{-6} cm/s (the $P_{app}$ estimated for guanidine on the basis of its molecular weight),31 passive diffusion of guanidine in the ms direction probably occurs predominantly via the paracellular transport pathway. This is likely the exclusive pathway for guanidine transport in the sm direction.

Kinetic evaluation of guanidine transport in the conjunctiva over 0.02 to 10 mM yielded a $K_{m}$ of 3.1 mM and a $J_{max}$ of 11.4 nmol/(cm$^2$ h) (Fig. 2). The $K_{m}$ of 3.1 mM is in a range of that for guanidine uptake via a H+ gradient-dependent process in renal (3.4 mM)$^{32}$ and placental (2.5 mM)$^{33}$ brush-border membranes and via an inside-negative membrane potential-dependent transport process in HeLa cells (1.5 mM).$^{34}$ The $J_{max}$ of 11.4 nmol/(cm$^2$ h), on the other hand, is comparable to that for monocarboxylate [8.9 nmol/(cm$^2$ h)]$^{20}$ and for glucose [39.2 nmol/(cm$^2$ h)]$^{29}$ transport systems in the conjunctiva. Various amines such as epinephrine (4.4 nM)$^{35}$ nor epinephrine (3.7 nM)$^{35}$ dopamine (58 nM)$^{17}$ histamine (90 nM), a secondary amine (dipivefrine), a tertiary amine (propanamide), quaternary amines (TEA, choline, and carbachol), heterocyclic amines (clonidine and brimonidine), and basic amino acids (D-arginine and L-arginine). Guanidine $P_{app}$ was significantly inhibited by all the OC compounds tested ($P < 0.05$), notably by amiloride, TEA, and choline (>80%). Interestingly, the guanidine $P_{app}$ in the ms direction was decreased by the antiglaucoma drugs dipivefrine (72%), brimonidine (70%), and carbachol (78%). In the case of TEA, its $P_{app}$ was significantly inhibited by unlabeled TEA and guanidine at 1 mM by 94% and 47%, respectively (Table 2).

**Table 1. Effects of Pharmacological Agents on Guanidine Transport in the ms and sm Directions in the Excised Pigmented Rabbit Conjunctiva**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ms</th>
<th>sm</th>
<th>Net</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.93 ± 0.12</td>
<td>0.64 ± 0.07</td>
<td>1.30 ± 0.14† (100)</td>
</tr>
<tr>
<td>+0.5 mM ouabain</td>
<td>1.71 ± 0.23</td>
<td>0.68 ± 0.06</td>
<td>1.03 ± 0.24† (79)</td>
</tr>
<tr>
<td>+1 μM valinomycin</td>
<td>1.30 ± 0.14*</td>
<td>0.92 ± 0.18</td>
<td>0.38 ± 0.25* (29)</td>
</tr>
<tr>
<td>+1 μM valinomycin (116.3 mM K+ on the mucosal side)</td>
<td>1.16 ± 0.23*</td>
<td>0.92 ± 0.07</td>
<td>0.24 ± 0.24* (18)</td>
</tr>
<tr>
<td>+0.1 mM monensin</td>
<td>2.50 ± 0.40</td>
<td>0.98 ± 0.19</td>
<td>1.51 ± 0.44† (116)</td>
</tr>
<tr>
<td>+0.3 μM FCCP</td>
<td>2.02 ± 0.09</td>
<td>0.72 ± 0.22</td>
<td>1.30 ± 0.24† (100)</td>
</tr>
</tbody>
</table>

All experiments were conducted in the presence of 1 μCi/ml [14C]guanidine and 0.1 mM unlabeled guanidine. All pharmacological agents were added to both bathing fluids, unless noted otherwise. Data represent means ± SEM (n = 3–6). The numbers in parentheses in the “net” column are percentage of control of the net $P_{app}$ ($P_{app,ms} - P_{app,sm}$).

* $P < 0.05$, significantly different from control.
† $P < 0.05$, significantly different from zero.
nM), serotonin (15 nM) exist in the tear fluid at concentrations below the estimated \( K_m \) for guanidine transport. Although it is tempting to speculate that the conjunctival OC transport process may play a role in scavenging these amines, lacrimal secretion may be more important in maintaining their tear concentration.

**Driving Force for Conjunctival Guanidine Transport**

The OC transport process in the conjunctiva might be of an inside-negative membrane potential-dependent type. This is indicated by elimination of net guanidine transport by valinomycin (Table 1). Moreover, as is the case for other membrane potential-dependent OC transport in the placenta and renal proximal tubules, a decrease in extracellular pH from 7.4 to 7.0 caused a depolarization of cell membrane potential from \(-60 \) to \(-40 \) mV, thereby reducing OC transport. Furthermore, the transport system was able to recognize both TEA and choline (Fig. 4, Table 2). Thus, the transport system in the conjunctiva does not fit the profile of the OCT3, OCTN2, or OC\(_1\)/H\(_1\) exchange type. Moreover, because conjunctival net guanidine transport was not significantly affected by monensin or serosal ouabain treatment (Table 1), the conjunctival OC transport process is not likely to be an Na\(^+\)-dependent active transport process. Although ouabain may induce depolarization of the cell membrane potential, this may not be sufficient to abolish membrane potential–dependent solute transport. Ouabain treatment at 0.1 mM for 2 hours has been reported to depolarize an inside-negative membrane potential by 3% in *Aplysia* intestinal epithelial cells and by 18% in toad bladder epithelial cells.

**Substrate Specificity for the Conjunctival OC Transport Process**

Notable differences in substrate specificity are known to exist among the OC transport systems. For example, whereas TEA is recognized by all OC transporters, choline is not a substrate for OCT3 and OCTN2. Moreover, neither choline nor TEA inhibited guanidine uptake via an OC\(_1\)/H\(_1\) antiport system in the placenta. In the conjunctiva, we found that the OC transport system was able to recognize both TEA and choline (Fig. 4, Table 2). Thus, the transport system in the conjunctiva does not fit the profile of the OCT3, OCTN2, or OC\(_1\)/H\(_1\) type. Further investigation will be needed to confirm the substrate specificity of the conjunctival OC transport process.

**TABLE 2. Effect of Unlabeled TEA and Guanidine on \(^{14}C\)TEA Transport Across the Pigmented Rabbit Conjunctiva in the ms Direction**

<table>
<thead>
<tr>
<th>TEA ( P_{app} (\times 10^{-6} \text{ cm/s}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+1 mM TEA</td>
</tr>
<tr>
<td>+1 mM Guanidine</td>
</tr>
</tbody>
</table>

Data represent means ± SEM (n = 3–6).

\* \( P < 0.05 \), significantly different from control.
cations in L- and D-arginine that contributes to sub-strate interaction with the OC transporter.

**Possible Role of OC Transport Process in the Conjunctival Transport of Cationic Ophthalmic Drugs**

Certain OC type antiglaucoma drugs may use the OC transport system to gain access to the underlying ocular tissue. Indeed, guanidine transport in the conjunctiva was significantly inhibited by dipivefrine, brimonidine, and carbachol by 72%, 70%, and 78% (Fig. 4), respectively. Acheampong et al.\(^4^4\) reported that 10 minutes after the topical instillation of 35 μL of a 0.5% (11.3 mM) brimonidine tartrate solution to the pigmented rabbit eye, drug concentrations in the iris-ciliary body and aqueous humor reached 10.3 and 2.1 nmol/g, respectively. This is consistent with the 18.7 nmol of drug traversing the conjunctiva, as estimated from the total flux of 28.1 nmol/aqueous humor reached 10.3 and 2.1 nmol/g, respectively. (11.3 mM) brimonidine tartrate solution to the pigmented

### References


45. Serle JB. A comparison of the safety and efficacy of twice daily brimonidine 0.2% versus betaxolol 0.25% in subjects with elevated intraocular pressure. *Surv Ophthalmol.* 1996;41:S39–S47.