Transport Mechanisms of Enoxacin in Rat Brush-Border Membrane of Renal Cortex: Interaction with Organic Cation Transport System and Ionic Diffusion Potential Dependent Uptake

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The mechanism of the renal transport of enoxacin (ENX) has been investigated using brush-border membrane vesicles (BBMVs) isolated from the rat renal cortex. The initial rate and time-course of ENX uptake were quite dependent upon the medium pH (pH 5.5 > pH 7.5). The pH dependence was in accordance with the degree of cationic form. Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) affected the transient uphill transport of ENX across the renal brush-border membrane in the presence of an outward-directed H⁺-gradient. The initial uptake was saturable, and transport kinetic parameters were given for a Kₘ and Vₘₐₓ of 0.59 nmol/mg protein/30 s, respectively. On the other hand, an outward H⁺-gradient (pHₖᵢₙ = 5.5, pHₘᵢₓ = 7.5) dependent uptake of ENX was partially decreased by the voltage-clamped BBMVs. Furthermore, a valinomycin-induced K⁺-diffusion potential (interior negative) was found to increase the uptake of ENX at pH 5.5, which is cationic form-rich. These results suggest that ENX uptake participates in not only the H⁺/organic cation antiport system for organic cation secretion but also the ionic diffusion potential (interior negative) dependent permeation through the membrane.

Key words enoxacin (ENX); brush-border membrane (BBM); membrane transport; H⁺/organic cation antiport; membrane potential; renal proximal tubule

Enoxacin (ENX) is an oral fluoroquinolone antibacterial agent beneficial in the treatment of a wide range of bacterial infections. Our previous studies demonstrated that the cationic form of this antibacterial agent was transported via a membrane potential-dependent permeation route for organic cations in intestinal brush-border membranes. ENX has two pKₐ values (pKₑ₁ = 6.2; anionic charge, and pKₑ₂ = 8.8; cationic charge), and is ionized by a cation or zwitterion at physiological pHs. Therefore, the transport behavior of the cationic form appeared to play a stronger role in the intestinal absorption process because the pH in the vicinity of intestinal epithelial cells is weakly acidic (pH 5–6).

With regard to the renal handling of ENX, several pharmacokinetic studies have suggested its urinary excretion may be through both tubular secretion and glomerular filtration. Okano et al. reported that ofloxacin, which is also a new quinolone, interacted with the H⁺/organic cation antiporter system related to tubular secretion in renal brush-border membranes. However, they failed to detect the countertransport effect of ofloxacin on [¹⁴C]tetraethylammonium uptake. Further, uptake experiments of organic cations gave evidence that overshoot-uptake generated by the diffusion potential (inside-negative) and mutual inhibition between organic cation were also observed in liposomes lacking the carrier-proteins. The present study was designed to obtain firm evidence concerning the transport mechanisms underlying the secretion of ENX by the proximal tubules of the kidney.

MATERIALS AND METHODS

Materials ENX was kindly donated by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Valinomycin, N¹-methylnicotinamide, disopyramide and carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Guanidine, cimetidine and tetraethylammonium were obtained from Nacalai Tesque Inc. (Kyoto, Japan), Aldrich Chemical Co. (Wisconsin, U.S.A.), and Wako Pure Chemical Ind. (Osaka, Japan), respectively. All other chemicals were of the highest grade available and were used without further purification.

Preparation of Renal Brush-Border Membrane Vesicles (BBMVs) BBMVs were isolated from the renal cortex of male Wistar rats (200–250 g) by the calcium precipitation method according to Evers et al. Unless otherwise specified, final pellet brush-border membranes were suspended in an experimental buffer containing 100 mm D-mannitol, 100 mm salts (KCl, K gluconate, Na gluconate) and either 20 mm HEPES-Tris (pH 7.5) or 20 mm MES-Tris (pH 5.5). The purity of the membrane vesicles was routinely evaluated by the enrichment of alkaline phosphatase, the enzymes specific to the renal brush-border membrane. The specific activities of these enzymes increased about 11 fold in the final membrane suspension compared with the concentration found in the homogenate of the renal cortex.

Uptake Experiments The uptake of substrates (0.5 mm) by the freshly isolated membrane vesicles was measured at 25 °C by a rapid filtration technique using a Millipore Filter (HAWP, 0.45 μm, 2.5 cm diam.) which was pre-treated with 0.3% polyethyleneimine to avoid nonspecific adsorption to the filter, as described previously. As a blank, a membrane-free incubation medium was handled in an identical manner.

Analytical Methods The concentration of ENX was measured...
determined by high-performance liquid chromatograph (Hitachi L-6000) equipped with an L-4000 UV detector (Hitachi, Ltd., Tokyo, Japan) as described in a previous report.\textsuperscript{10} Separation was achieved on a reversed phase column (Inertsil ODS, 5 μm, 4 mm i.d. × 250 mm) using a mobile phase consisting of methanol: 0.05 M KH₂PO₄ containing 2% acetic acid (3:7) at a flow rate of 0.9 ml/min. The protein concentration was determined by the method of Lowry et al.\textsuperscript{11} with bovine serum albumin as a standard.

RESULTS

Effect of the Medium pH on ENX Uptake by Rat Renal BBMVVs The uptake of ENX was studied in different buffers of various pH (pH\textsubscript{in} = pH\textsubscript{out}). As shown in Fig. 1, the uptake behavior of ENX at pH 5.5 was greater than those at pH 6.5 and pH 7.5, indicating that binding of ENX to renal BBMVVs can be associated with the degree of ionization to cationic forms. The pH effect on the ENX binding to membrane vesicles was clarified by measuring the uptake of ENX by the membrane vesicles after a 30 min incubation period while varying the medium osmolarity (Fig. 1, right). The values of ENX binding estimated by extrapolation to infinite osmolarity were distinctly dependent upon the medium pH, and the rank order of binding at each pH was in agreement with that of uptake behavior. ENX binding at pH 5.5 was greater than that at pH 7.5, therefore indicating that the cationic form of ENX interacts with the surface of renal brush-border membranes.

Effect of H⁺-Gradient on ENX Uptake Figure 2 shows the effect of an outwardly directed proton gradient on ENX uptake by rat renal BBMVVs. In the presence of such a gradient (pH\textsubscript{in} = 5.5; pH\textsubscript{out} = 7.5), there was a transient accumulation of ENX inside the vesicles above the equilibrium value, demonstrating an uphill transport. The intravesicular concentration of ENX at the peak of overshoot was approximately three times the equilibrium value. This proton gradient-stimulated ENX uptake was not due to the influence of the proton gradient on the intravesicular space, since the equilibrium value of ENX uptake which depends on the intravesicular volume remained almost the same in the presence or absence of the proton gradient. The insert of Fig. 2 illustrates the earlier time-course on the uptake of ENX in the presence of an outward proton gradient. The initial uptake of ENX increased as a function of elapsed time, indicating uptake inside the membrane vesicles. Furthermore, in the presence of a protonophore, FCCP, a transient accumulation of ENX inside the vesicles was markedly decreased following the dissipation of the proton gradient by the FCCP (Fig. 2).

In contrast, ENX uptake in the presence of an inwardly directed H⁺-gradient was significantly increased by the addition of FCCP, though an uphill transport of ENX occurred neither in the presence nor absence of FCCP.
ENX Uptake by Voltage-Clamped Renal BBMVs To elucidate the effect of an outward-directed H⁺-gradient on the uptake of ENX into the membrane vesicles, uptake was examined by normal or voltage-clamped renal BBMVs in the presence of an outward H⁺-gradient. Under these experimental conditions, K⁺ was present in equimolar concentrations both inside and outside the vesicles, while valinomycin, a K⁺ ionophore, was added to the vesicle suspension beforehand. Therefore, only the H⁺-diffusion potential was instantly compensated by K⁺ movement while the H⁺-gradient itself remained. The voltage-clamped BBMVs of rat kidney have exhibited a significant decrease in the overshoot phenomenon in the presence of an outward proton gradient, although this has not been total (Fig. 4).

Effect of an Outwardly Directed K⁺-Diffusion Potential To clarify in further detail the role of the transmembrane ionic diffusion potential, the effect of a valinomycin-induced K⁺-diffusion potential on ENX uptake in renal BBMVs was investigated. As shown in Fig. 5, the initial uptake of ENX by renal BBMVs was significantly stimulated in the presence of the K⁺-diffusion potential (interior negative).

Kinetic Analysis of H⁺/ENX Antiport System in Renal Brush-Border Membrane To determine the effect of increased concentration of ENX on its uptake in the presence of an outward H⁺-gradient, the initial (30 s)...
uptakes were determined by measuring the uptake with a range of concentrations from 50 μM to 500 μM. Figure 6 illustrates that the uptake was saturable as a function of ENX concentration. In these experiments, ENX uptake at the equilibrium (pH_{in} = 7.5) was used to determine the nonsaturable diffusion component at each substrate concentration. The rate of carrier-mediated uptake, which was determined by subtracting the diffusion component from the total uptake, was adequately described by Michaelis–Menten kinetics (Fig. 6a). A Lineweaver–Burk transformation of the corrected data resulted in values for the apparent K_{m} (affinity constant) and V_{max} (maximum velocity) of 0.59 mM and 1.37 nmol/mg protein/30 s, respectively (Fig. 6b).

**Inhibitory Effect of Organic Cations on H^{+}-Gradient Dependent Uptake of ENX** We investigated the inhibitory effects of several organic cations on the H^{+}-gradient dependent uptake of ENX in renal brush-border membrane vesicles (Fig. 7). The uptake rates for ENX were measured with 30 s and 30 min incubation periods in the presence of an outward H^{+}-gradient. The typical substrates of the renal H^{+}/organic cation antiporter, such as N^{1}-methylnicotinamide (NMN), tetraethylammonium (TEA), and cimetidine, had slight inhibition, although not significant, on the ENX uptake with a 30 s incubation period even in the presence of an outward H^{+}-gradient. Disopyramide and guanidine, on the other hand, significantly inhibited ENX uptake by the membrane vesicles. The equilibrium values of ENX uptake with a 30 min incubation period remained almost the same in the presence or absence of all other cations.

**DISCUSSION**

Our previous studies\(^7\)–\(^9,\)\(^13\) indicated that the uptakes of several organic cations into the intestinal BBMVs were stimulated by the ionic diffusion potential, and the cationic form of ENX was also taken up across the intestinal brush-border membrane by the H^{+}-diffusion potential (interior negative), but not the carrier-mediated process. In this study, there are similarities between intestinal brush-border membranes and renal brush-border membranes with respect to the presence of a membrane potential dependent pathway for ENX, since both a reduction of the ENX uptake in the voltage-clamped BBMVs and a stimulation effect of the K^{+}-diffusion potential on the uptake were observed.

The present results also demonstrated that ENX can be transported via an H^{+}-antiport system in rat renal BBMVs. A transient accumulation of ENX observed in response to the outward H^{+}-gradient was distinctly decreased in the presence of FCCP. Moreover, an inward H^{+}-gradient reduced the initial uptake rate of ENX by the renal BBMVs. These results show that this fluoroquinolone is transported across the brush-border membrane coupling with H^{+} itself on the opposite side of the membrane bilayer.

We have confirmed that the stimulative uptake of disopyramide by an outward H^{+}-gradient was associated with an H^{+}-antiport system in renal brush-border membranes, whereas, in the intestinal brush-border membrane, its stimulation was caused by the effect of an interior negative H^{+}-diffusion potential.\(^13\) In the present study, ENX uptake by renal BBMVs seemed to be sensitive to both the H^{+}-gradient itself and the H^{+}-diffusion potential (inside-negative), due to the fact that its overshoot uptake generated by the outward H^{+}-gradient was not quite eliminated even in the voltage-clamped BBMVs. Also, as shown in Fig. 3, the final ENX uptake by BBMVs was still lower in the presence of an inward H^{+}-gradient despite the final pH of medium being close to 5.5. The inwardly directed H^{+}-diffusion potential (inside-positive) may have an inhibitory effect on the influx of ENX into the renal BBMVs.

The saturable process for ENX uptake obtained from this study contained components of both the H^{+}-antiport system and H^{+}-diffusion potential sensitive permeation. However, H^{+}-gradient dependent uptake of ENX at a concentration of 0.5 mM was almost FCCP-sensitive (Fig. 2), and the kinetic parameters given in the present study (K_{m}=0.59 mM, V_{max}=1.37 nmol/mg protein/30 s) were approximately in agreement with those for TEA and guanidine uptake.\(^13\) Therefore, the H^{+}-diffusion potential dependent component of ENX uptake is considered.
to be extremely small under this experimental condition (0.05—0.5 mM ENX).

The uptake of ENX by the renal BBMVs was significantly inhibited by disopyramide and guanidine, although other cations such as TEA, NMN, and cimetidine caused only slight inhibition. Several investigators\(^{14,15}\) mentioned that renal brush-border membranes possessed more than one H\(^+\)/organic cation antiporter for the handling of organic cations. Miyamoto et al.\(^{15}\) found that an H\(^+\)/organic cation antiport system for guanidine in renal brush-border membrane was distinct from the H\(^+\) antiporter available for TEA and NMN in this membrane. On the other hand, Ullrich\(^{10}\) described recently that H\(^+\)/organic cation antiporters in the renal brush-border membrane could be classified into three groups by the substrate specificity, and that the H\(^+\)/cimetidine antiport system was different from the H\(^+\)/organic cation antiporter for TEA, NMN, and guanidine. Therefore, although there may be a necessity to investigate in more detail the substrate specificity for an H\(^+\)-antiport system among these compounds, the present results suggest that ENX can be recognized as a substrate for the H\(^+\)-antiporter for the organic cations such as guanidine and disopyramide.

In conclusion, the present study gives evidence that ENX transport in rat renal brush-border membranes is mediated by at least two transport systems; one is an H\(^+\)/ENX antiport system, and the other is an outward H\(^+\)-diffusion potential-dependent permeation system of organic cations similar to that in the intestinal brush-border membrane.

REFERENCES