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The pH Dependent Uptake of Enoxacin by Rat Intestinal Brush-border Membrane Vesicles

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Abstract—The mechanism of the intestinal transport of enoxacin, an orally active fluoroquinolone antibiotic, has been investigated using brush-border membrane vesicles isolated from rat small intestine. The initial rate and time-course of enoxacin uptake were considerably dependent upon the medium pH (pH 5·5 > pH 7·5) and upon the percent ionization of the carboxyl group (pK_a 6·2, anionic charge), namely, the degree of uptake of cationic form was higher than that of the zwitterionic form. There was evidence of transport into the intravesicular space as shown by the effect of extravesicular medium osmolarity on enoxacin uptake at steady state (30 min). This transport across the brush-border membrane was stimulated by the valinomycin-induced K^+-diffusion potential (interior negative) and an outward H^+-diffusion potential. Furthermore, changing the pH of the medium from 5·5 to 7·5 significantly decreased the effect of valinomycin-induced K^+-diffusion potential on the enoxacin uptake. These results suggest that the uptake behaviour of the cationic form of enoxacin plays an important role in the intestinal absorption process of enoxacin.

Enoxacin is a new, orally active, synthetic broad-spectrum antibacterial agent of the fluorinated quinolone class. This drug was rapidly absorbed after oral administration as judged from the time required to reach the maximum plasma concentration within 0·5 to 3 h (Wise et al 1986; Somogyi et al 1987; Somogyi & Bochner 1988). Because this drug is sparingly water soluble and exists as a zwitterion with both basic and acidic functional groups, the rapid absorption profile is somewhat unusual but is consistent with that observed with other drugs, such as aminocephalosporins, which have similar physicochemical properties. Whether passive diffusion is the sole mechanism for the gastrointestinal absorption of enoxacin is unknown. Okezaki et al (1988) have reported that the distribution of quinolones into various tissues was very rapid and their tissue-to-plasma concentration ratios, especially in the liver and kidney, were considerably larger than those of inulin (an extracellular fluid marker). There is also kinetic evidence that lomefloxacin and ofloxacin, once diffused into cerebrospinal fluid (CSF), may be sequestered from CSF to blood via an unknown transport system in the choroid plexus (Sato et al 1988). Moreover, Prieto et al (1988) reported evidence of a saturable process for the intestinal absorption of ofloxacin. However, the detailed mechanism of the membrane transport of quinolones has not yet been clarified, and there exists little information available on the handling of enoxacin by the intestinal brush-border membrane.

Regarding the membrane transport of zwitterionic compounds, some investigators have reported that cephradine, which is also a zwitterionic antibiotic may be transported via a dipeptide carrier in rabbit small intestine (Okano et al 1986; Inui et al 1988; Kramer et al 1988; Kato et al 1989). However, recently, we have found little contribution by the dipeptide carrier system to the uptake of zwitterionic cephalosporins into rat and human intestinal brush-border membrane vesicles (Iseki et al 1989; Sugawara et al 1991a,b).

The present investigation was designed to examine the absorption mechanisms of enoxacin (Fig. 1) in rat intestinal brush-border membranes using intestinal brush-border membrane vesicles (Knickelbein et al 1983; Murer et al 1984; Semenza et al 1984; Iseki et al 1985, 1988, 1991; Worman & Field 1985; Bluett et al 1986; Rajendran et al 1987; Sugawara et al 1990).

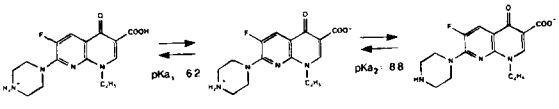

FIG. 1. Chemical structure and ionization of the quinolone antibacterial agent, enoxacin.

Materials and Methods

Materials

Enoxacin was kindly donated by Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Pipemidic acid and valinomycin were purchased from Sigma Chemicals (St Louis, MO, USA). All other chemicals were of the highest grade available and were used without purification.

Preparation of the brush-border membrane vesicles and uptake experiments

Adult male Wistar rats, 180–240 g, were used. The entire small intestine was excised under ether anaesthesia and brush-border membrane vesicles were prepared by $CaCl_2$ precipitation (Kessler et al 1978) as described by Iseki et al (1989). 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%

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polyethylenimine to avoid the nonspecific adsorption by the membrane filter.

In a standard assay, the uptake was initiated by the addition of 200 μL of the experimental buffer containing enoxacin (0·6 mM) to 40 μL of membrane vesicle suspension in a plastic tube. The specific conditions for each experiment are given in the figure legends. At a stated time, the reaction was halted by dilution of an incubation sample with 3 mL of ice-cold buffer (150 mM NaCl and either 10 mM HEPES-Tris, pH 7·5 or 10 mM Mes-Tris, pH 5·5) followed by rapid filtration through a membrane filter. The filter was washed once with 5 mL of the same ice-cold buffer. The enoxacin trapped on the filter was extracted with 400 μL of 0·1 M acetic acid in a plastic vial to avoid the adsorption of enoxacin to the glass surface and the concentration of extracted enoxacin determined by HPLC. As a blank, membrane-free incubation medium was handled in an identical manner.

Analytical methods

The concentration of enoxacin was determined by HPLC (Hitachi L-6000) equipped with an L-4000 UV detector (Hitachi Ltd, Tokyo, Japan) with the wavelength set at 265 nm. 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 KH_2PO_4 containing 2% acetic acid (3:7) at a flow rate of 0·9 mL min^{-1}. The limit of detection was 3 pmol for enoxacin. Pipemidic acid was used as an internal standard. Protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as standard.

Results

Transport of enoxacin in the presence of ionic gradient

The viability of isolated membrane vesicles was ascertained by examining the intact active transport of D-glucose and L-alanine into vesicles against a concentration gradient in the presence of an inward Na^+ gradient (Iseki et al 1989; Sugawara et al 1990). The effect of a Na^+ gradient on D-glucose uptake was similar to that reported previously (Iseki et al 1989) (data not shown). The results of the effects of a Na^+ and H^+ gradient on the uptake of enoxacin into the membrane vesicles are shown in Figs 2, 3 and 4.

There was an increase in enoxacin uptake with time, but the effect of an inward Na^+ gradient on enoxacin uptake was not observed (Fig. 2). Moreover, as shown in Fig. 3, there was no stimulation or overshoot of enoxacin transport at early time points in the presence of the proton gradient ($pH_{in} = 7·5$; $pH_{out} = 5·5$). On the other hand, as indicated in Fig. 4, enoxacin uptake was relatively rapid in the presence of an outwardly directed H^+ gradient ($pH_{in} = 5·5$; $pH_{out} = 7·5$) and exhibited a stimulated uptake at the relatively early time points.

Effect of the medium pH on enoxacin uptake

Depending on the pH of the medium, enoxacin will be present in various amounts of the cationic or zwitterionic form. We investigated the influence of the medium pH (5·5 and 7·5) on enoxacin uptake at the same extra- and intravesicular medium pH. As shown in Fig. 5, the time-course of enoxacin uptake at pH 7·5 was significantly reduced compared with that at pH 5·5.

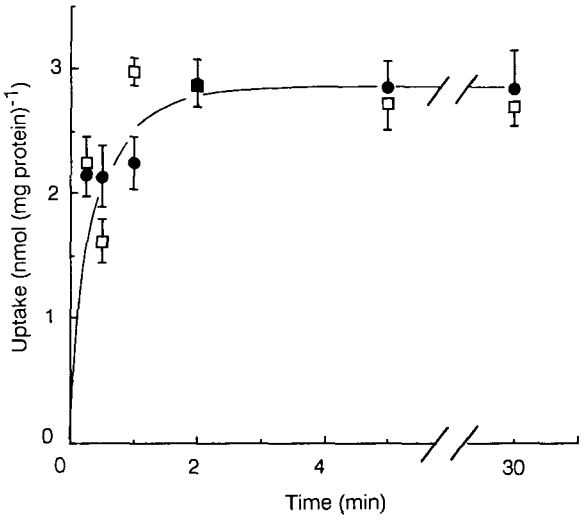

FIG. 2. Effect of Na^+ gradient on enoxacin uptake by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM KCl, 100 mM D-mannitol and 20 mM Mes-Tris buffer (pH 5·5). The vesicles (40 μL) were incubated with 200 μL of 20 mM Mes-Tris buffer (pH 5·5), containing 0·6 mM enoxacin, 100 mM D-mannitol and either 100 mM NaCl (□) or 100 mM KCl (●). Results represent the means ± s.e.m. of 3–6 measurements with different preparations of vesicles.

Fig. 6 indicates the effect of the medium pH (5–8) on the initial uptake of enoxacin and the extent of ionization of the carboxyl group of enoxacin. The initial rate of uptake, as measured up to 0·5 min, was greater at the lower pH environment than at the neutral pH. The shape of the graph

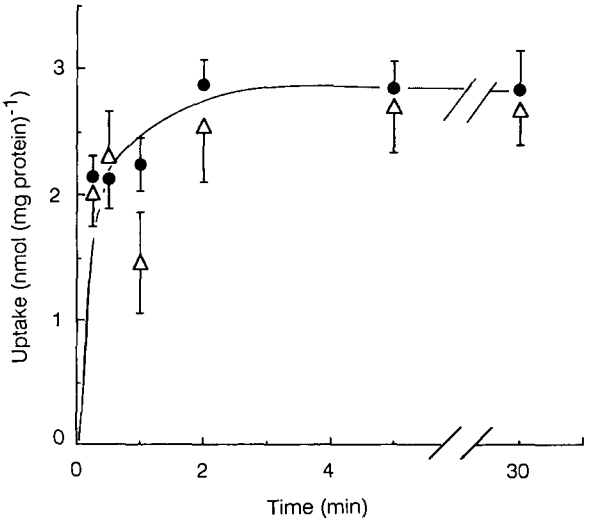

FIG. 3. Effect of an inward H^+ gradient on enoxacin uptake by rat intestinal brush-border membrane vesicles. Membrane suspensions (40 μL) were preloaded in 100 mM KCl, 100 mM D-mannitol and either 20 mM HEPES-Tris buffer (pH 7·5) (△), or 20 mM Mes-Tris buffer (pH 5·5) (●). Transport studies were performed by adding incubation medium (200 μL) containing 20 mM Mes-Tris buffer (pH 5·5), 100 mM KCl, 100 mM D-mannitol and 0·6 mM enoxacin. Results represent the means ± s.e.m. of 5–10 determinations made on the different preparations of vesicles.

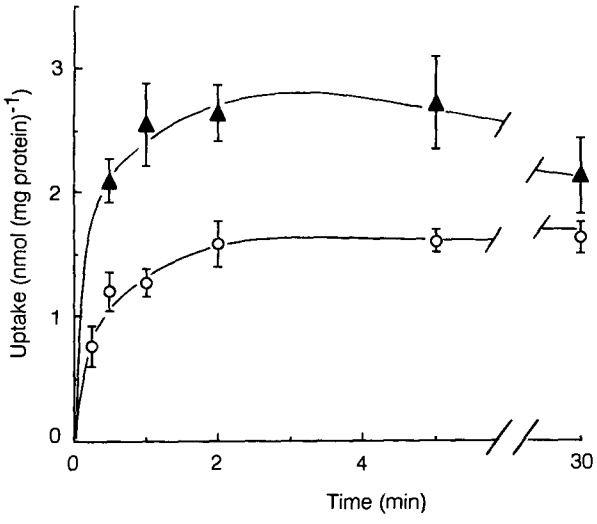

FIG. 4. Effect of an outwardly directed H^+ gradient on enoxacin uptake by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM KCl, 100 mM D-mannitol and either 20 mM HEPES-Tris buffer (pH 7·5) (○) or 20 mM Mes-Tris buffer (pH 5·5) (▲). Transport studies were performed by adding an incubation medium containing 100 mM KCl, 100 mM D-mannitol, 0·6 mM enoxacin and 20 mM HEPES-Tris buffer (pH 7·5). Final concentration of enoxacin in the incubation media was 0·5 mM. Results represent the means ± s.e.m. of 6–10 measurements with different preparations of vesicles.

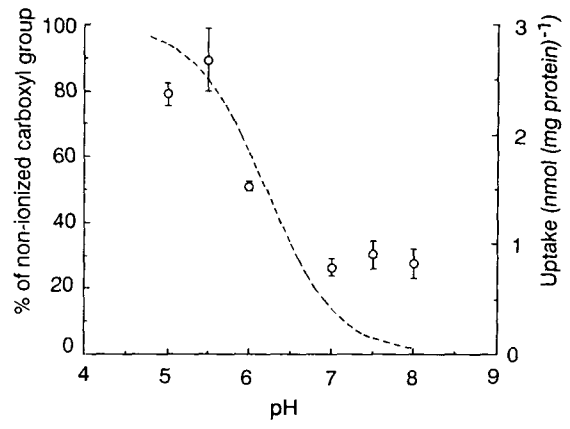

FIG. 6. Correlation of pH of the incubation media with the initial (30 s) uptake of enoxacin by the brush-border membrane vesicles of rats. The uptake studies were performed in the media containing 0·5 mM enoxacin, 100 mM KCl, 100 mM D-mannitol and either 20 mM HEPES-Tris buffer (pH 7–8) or 20 mM Mes-Tris buffer (pH 5–6). Each point represents the mean ± s.e.m. of 3–6 measurements with different preparations of vesicles. The dotted line indicates the theoretical profile of ionization of the carboxyl group of enoxacin in aqueous solution as a function of pH.

of the uptake behaviour was in agreement with the profile of the ionization of the carboxyl groups of enoxacin, with the maximum change in uptake coinciding with the pK_a value (6·2) of the carboxyl group of enoxacin.

In order to clarify the detail of pH-dependency of the medium, the effect of the extravesicular osmolarity on the uptake of enoxacin was investigated. Although the uptake of enoxacin decreased with increasing medium osmolarity at both pH 5·5 and 7·5, there was a distinct pH-dependency of the binding (estimated by extrapolation to infinite osmolarity) to the brush-border membrane surface (Fig. 7). This result suggested that the greater uptake of enoxacin at pH 5·5 (Fig. 5) is due in part to the differences in binding at pH 5·5 and 7·5.

Effects of K^+-diffusion potential and an outward H^+ gradient on enoxacin uptake

The greater uptake of enoxacin observed in the presence of an outwardly directed H^+ gradient (Fig. 4) may be due to the

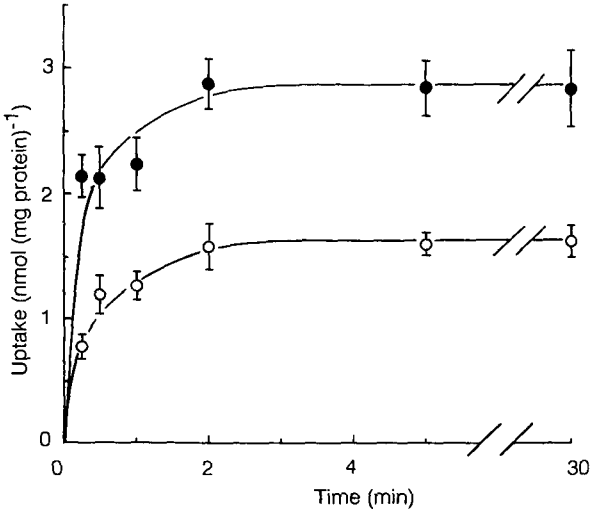

FIG. 5. Effect of the medium pH on enoxacin uptake by the intestinal brush-border membrane vesicles. The vesicles were assayed in the medium containing 0·5 mM enoxacin, 100 mM KCl, 100 mM D-mannitol and either 20 mM HEPES-Tris buffer (pH 7·5) (○) or 20 mM Mes-Tris buffer (pH 5·5) (●) without H^+ gradient. Results represent the means ± s.e.m. of 5–10 measurements with different preparations of vesicles.

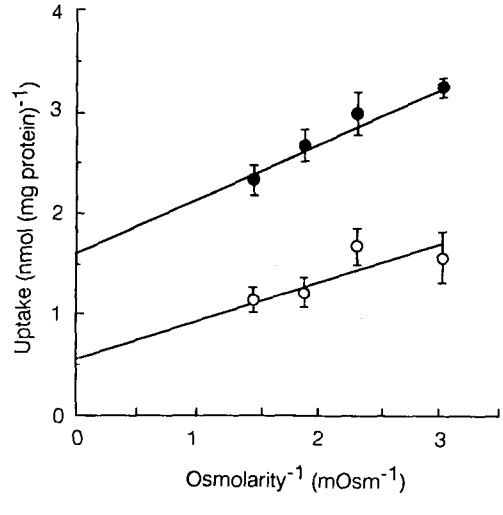

FIG. 7. Uptake of enoxacin as a function of osmolarity of the incubation medium. Uptake experiments were performed in the medium containing either 20 mM HEPES-Tris buffer (pH 7·5) (○) or 20 mM Mes-Tris buffer (pH 5·5) (●), 0·5 mM enoxacin, 100 mM KCl and various concentrations of D-cellobiose to give the desired medium osmolarity. Values represent the means ± s.e.m. of 8 determinations from the different preparations at 30 min incubation.

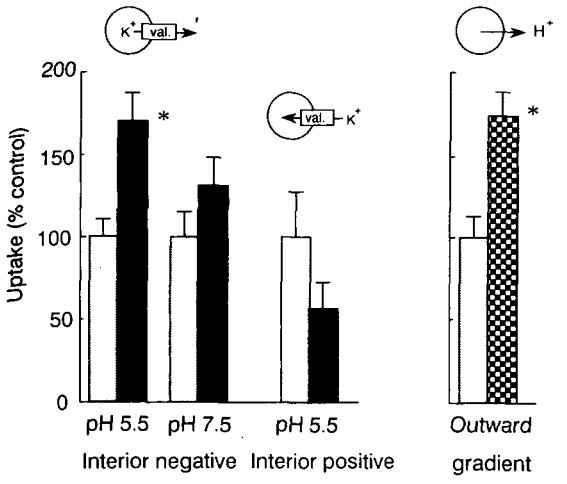

FIG. 8. Effects of valinomycin (val.)-induced K^+-diffusion potential and H^+-diffusion potential on enoxacin uptake (15 s) by rat intestinal brush-border membrane vesicles. To produce the interior negative K^+-diffusion potential, membrane vesicles were equilibrated with 100 mM D-mannitol, 100 mM potassium gluconate, and either 20 mM HEPES-Tris buffer (pH 7·5) (inside negative, pH 7·5; lightly shaded bars, hatched bars) or 20 mM Mes-Tris buffer (pH 5·5) (inside negative, pH 5·5; lightly shaded bars, hatched bars). The uptake solutions were composed of either 20 mM HEPES-Tris buffer (pH 7·5) or 20 mM Mes-Tris buffer (pH 5·5), 100 mM D-mannitol, 100 mM sodium gluconate, 0·5 mM enoxacin and either valinomycin in ethanol yielding 3 μg (mg membrane protein)$^{-1}$ (hatched bars) or ethanol (the same volume as control) (lightly shaded bars). For the induction of the interior positive K^+-diffusion potential, the vesicles were suspended in a medium containing 100 mM sodium gluconate instead of potassium gluconate, and uptake studies were performed by diluting the drug solution containing 100 mM potassium gluconate with or without valinomycin. In the case of the effect of an outward H^+-diffusion potential on enoxacin uptake, the uptake study was performed as described in the legend to Fig. 3 (lightly shaded bars, control; chequered bar, in the presence of outward H^+ gradient). Data are expressed as means ± of 6 determinations made on the different preparations of vesicles.

effect of a cation diffusion potential in intestinal brush-border membranes due to a low H^+ conductance (Murer et al 1976). Valinomycin-induced K^+-diffusion potential would be expected to increase the uptake of enoxacin. As is evident in Fig. 8, a K^+-diffusion potential stimulated the initial uptake of enoxacin more at pH 5·5 than at pH 7·5. A positive diffusion potential inhibited the initial uptake of enoxacin (Fig. 8).

Discussion

The intestinal transport characteristics of enoxacin, investigated using rat small intestinal brush-border membrane vesicles, suggested that the pH-dependent binding of enoxacin to the membrane vesicles was associated with the dissociation state of the carboxyl group of enoxacin. Enoxacin has two pK_a values ($pK_{a_1} = 6{\cdot}2$, $pK_{a_2} = 8{\cdot}8$), and exists as the cation at pH 5·5 or the zwitterion at pH 7·5. The binding of the cationic form appeared to play a superior role in the process of intestinal absorption because the pH in the vicinity of intestinal epithelial cells is weakly acidic (pH 5–6) and proton-rich (Lucas et al 1976; Lucas 1983; Shimada & Hoshi 1987; Takuwa et al 1985).

Enoxacin transport into the intravesicular space across the brush-border membrane, could be stimulated pH-dependently by transmembrane potential difference, which was generated by a K^+-, or H^+-diffusion potential.

In a recent study, Okano et al (1990) have reported the interaction of ofloxacin with the organic cation-H^+ antiport system in rat renal brush-border membranes. In the kidney, this organic cation transport system was reported to be energized by an outwardly directed H^+ gradient (Holohan & Ross 1981; Inui et al 1985; McKinney & Kunnemann 1985, 1987; Rafizadeh et al 1986, 1987), and to be implicated in the active tubular secretion of ofloxacin by the kidney (Okano et al 1990) as a result of the pH of the proximal tubular fluid being acidic compared with the intracellular pH of the renal cell (Aronson 1983). There has been little indication as to whether such an H^+ antiport-carrier system is present in the small intestinal brush-border membrane, although Miyamoto et al (1988) reported that the transport system of guanidine in the small intestinal brush-border membrane was driven by an outwardly directed H^+ gradient. This pathway may explain the secretion to the intestinal lumen but not the absorption since the surface of the epithelial cell was weakly acidic and proton-rich. It is unlikely that the H^+ antiport-carrier system participates in the enoxacin transport from the intestinal lumen.

As indicated in Fig. 7, the interior negative transmembrane potential difference exhibited a significant effect on the uptake of enoxacin into the membrane vesicles, and uptake was significantly more efficient in an acidic, nongradient environment of pH 5·5 rather than that of pH 7·5. In contrast, the interior positive K^+-diffusion potential inhibited the uptake of enoxacin into the membrane vesicles. These results indicate that the transport process of enoxacin is electrophoretic, and suggest that the stimulation by an outwardly directed H^+ gradient might be based on the similar effect of interior negative transmembrane electrical potential. This effect of membrane potential difference on enoxacin uptake was similar to our previous studies with chlorpromazine and propantheline. These organic cations were driven into the brush-border membrane vesicles by the valinomycin-induced K^+-diffusion potential (Saitoh et al 1988, 1989).

In conclusion, we have shown that enoxacin uptake was stimulated by an outwardly directed H^+ gradient which was caused by the effect of an interior negative H^+-diffusion potential. Enoxacin transport was sensitive to changes in membrane potential, and enoxacin uptake was affected by the pH of the medium. This stimulant effect of acidic pH (5·5) on enoxacin uptake can be explained by the assumption that the cationic form of enoxacin is much better transported than the zwitterionic species, which is the predominant form at pH 7·5.

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