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Transport characteristics of grepafloxacin and levofloxacin in the human intestinal cell line Caco-2

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Abstract

Transport characteristics of grepafloxacin and levofloxacin across the apical membrane of Caco-2 cells were examined. Both grepafloxacin and levofloxacin uptakes increased rapidly, and were temperature-dependent. Grepafloxacin and levofloxacin uptakes showed concentration-dependent saturation with Michaelis constants of 3.9 and 9.3 mM, respectively. Uptake of grepafloxacin and levofloxacin increased in Cl^--free and ATP depleted conditions, suggesting the involvement of an efflux transport system different from the uptake mechanism. However, cyclosporin A, a typical inhibitor of P-glycoprotein, did not affect the uptake of these drugs. Unlabeled grepafloxacin, unlabeled levofloxacin and quinidine inhibited the uptake of grepafloxacin and levofloxacin under Cl^--free conditions. Tetraethylammonium, cimetidine, *p*-aminohippurate, probenecid, amino acids, β-lactam antibiotic or monocarboxylates did not inhibit the uptake of grepafloxacin and levofloxacin under the same conditions. In conclusion, our results suggested that grepafloxacin and levofloxacin uptakes were mediated by a specific transport system distinct from those for organic cations and anions, amino acids, dipeptides and monocarboxylates.

Keywords: Intestinal transport; Grepafloxacin; Levofloxacin; Caco-2 cell

1. Introduction

Quinolone antibacterial drugs are effective in the treatment of a variety of bacterial infections. Most of these drugs are well absorbed from the intestine, with bioavailability of close to 90%, and penetrate well into body tissues and fluids (Sörgel et al., 1989; Wolfson and Hooper, 1989). Prieto et al. (1988) reported that ofloxacin was absorbed by a carrier-mediated mechanism in a series of intestinal perfusion studies. Rabbaa et al. (1997) reported that ofloxacin absorption in rats was mediated by one or more intestinal transporters, distinct from dipeptide transporter but possibly some amino acid transporters. Conversely, sparfloxacin absorption was reported to be mediated by the dipeptide transport system in rats (Yamaguchi et al., 1991). Ciprofloxacin, enoxacin and sparfloxacin absorptions by rat intestinal brush-border membrane vesicles were reported to be potential-dependent (Iseki et al., 1992, 1998; Hirano et al., 1994, 1995). Thus, the mechanisms for absorption of quinolones were different between quinolones and experimental methods used.

The human colon adenocarcinoma cell line Caco-2 has been used as a model in which to study intestinal absorption or secretion of various drugs. This cell line spontaneously differentiates in culture into polarized cell monolayers with many enterocyte-like properties of transporting epithelia (Hidalgo et al., 1989). Caco-2 cells retain various transporters expressed in the intestine, for example, dipeptide transporter (Saito and Inui, 1993), amino acid transporter (McGivan and Pastor-Anglada, 1994), glucose transporter (Mesonero et al., 1994) and P-glycoprotein (Hunter et al., 1993). Using this model, a number of studies have been performed to characterize the intestinal transport of quinolones. Cormet et al. (1997) demonstrated that sparfloxacin was taken up across the apical membrane of Caco-2 cells by passive diffusion. Some investigators reported that transcellular transport of quinolones exhibited basolateral-to-apical flux corresponding to intestinal secretion, and that a carrier-mediated process was involved in the transport (Griffiths et al., 1993, 1994; Cavet et al., 1997). We examined the transcellular transport of grepafloxacin and levofloxacin in Caco-2 cells and showed

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that basolateral-to-apical flux of quinolones was mediated by P-glycoprotein and a specific transport system distinct from organic cation and anion transporters and multidrug resistance-associated protein (MRP) (Yamaguchi et al., 2000). However, little information is available concerning absorptive transport compared to secretory transport of quinolones in Caco-2 cells.

In this study, the transport characteristics of grepafloxacin and levofloxacin by Caco-2 cells were examined to characterize the intestinal absorption of quinolone antibacterial drugs. We investigated whether specific transport systems were involved in the uptake of grepafloxacin and levofloxacin across the apical membrane of Caco-2 cells and what kinds of transport systems contributed to the uptake of these drugs.

2. Materials and methods

2.1. Materials

D-[^{3}H]Mannitol (828.8 GBq/mmol) was purchased from NEN™ Life Science Products (Boston, MA). [^{14}C]Grepafloxacin (1.17 GBq/mmol) and unlabeled grepafloxacin were kindly supplied by Otsuka Pharmaceutical (Tokyo, Japan). [^{14}C]levofloxacin (1.07 GBq/mmol) and unlabeled levofloxacin were gifts from Daiichi Pharmaceutical (Tokyo). Cephalexin was obtained from Shionogi (Osaka). Tetraethylammonium, cimetidine, quinidine, *p*-aminohippurate, glycine, leucine, tryptophan, glutamate, lysine, β-alanine, γ-aminobutylic acid (GABA), lactate, salicylate and sodium azide were purchased from Nacalai Tesque (Kyoto). 2-Deoxy-D-glucose, probenecid and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS) were obtained from Sigma (St. Louis, MO). All other chemicals used were of the highest purity available.

2.2. Cell culture

Caco-2 cells at passage 18 obtained from the American Type Culture Collection (ATCC HTB37) were maintained by serial passage in plastic culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) as described previously (Inui et al., 1992). For uptake studies, Caco-2 cells were seeded on 35 mm plastic culture dishes at a density of 2.1×10^5 cells/cm^2 in 2 ml of culture medium. The medium consisted of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, MD) and 1% nonessential amino acid (Gibco) without antibiotics. The cells were grown in an atmosphere of 5% CO_2/95% air at 37 °C, given fresh medium every 3 or 4 days and used between the 20th and 21st day. In the present study, Caco-2 cells were used between passages 43 and 49.

2.3. Uptake studies

[^{14}C]Grepafloxacin and [^{14}C]levofloxacin uptakes were measured using monolayer cultures grown in 35 mm diameter dishes. The composition of incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.4) or 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 6.4, 7.4, 8.4). The pH of the medium was adjusted with a solution of HCl or NaOH. In Na^+-free medium, NaCl in HEPES-buffered saline was replaced with *N*-methyl-D-glucamine. In Cl^--free medium, NaCl, KCl, $CaCl_2$ and $MgCl_2$ in HEPES-buffered saline were replaced with respective gluconate salts. In high-K^+ medium, NaCl in HEPES-buffered saline was replaced with KCl. For ATP depleted condition, D-glucose in HEPES-buffered saline was replaced with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide. In general, uptake measurements were performed as described (Terada et al., 1997). D-[^{3}H]Mannitol (5 μM, 22.8 kBq/ml), a compound which is not transported by the cells, was used to calculate the extracellular trapping of [^{14}C]grepafloxacin (5 μM, 5.8 kBq/ml) and [^{14}C]levofloxacin (5 μM, 5.4 kBq/ml).

2.4. Protein assay

The protein contents of the cell monolayers solubilized in 1 N NaOH were determined by the method of Bradford (1976) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin as a standard.

2.5. Statistical analysis

Statistical significance of differences between mean values was calculated using the non-paired *t*-test. Multiple comparisons were performed using Scheffé's test after analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Uptake of grepafloxacin and levofloxacin by Caco-2 cells

As the first step, grepafloxacin and levofloxacin uptakes by Caco-2 cell monolayers were measured. Fig. 1 shows the time courses of the uptake of grepafloxacin and levofloxacin. Uptake of both quinolones was rapid, and almost reached a steady state at 15 min after the start of the incubation. The amount of grepafloxacin uptake at 30 min was fivefold greater than that of levofloxacin. Uptake of both quinolones was markedly decreased by lowering temperature (4 °C).

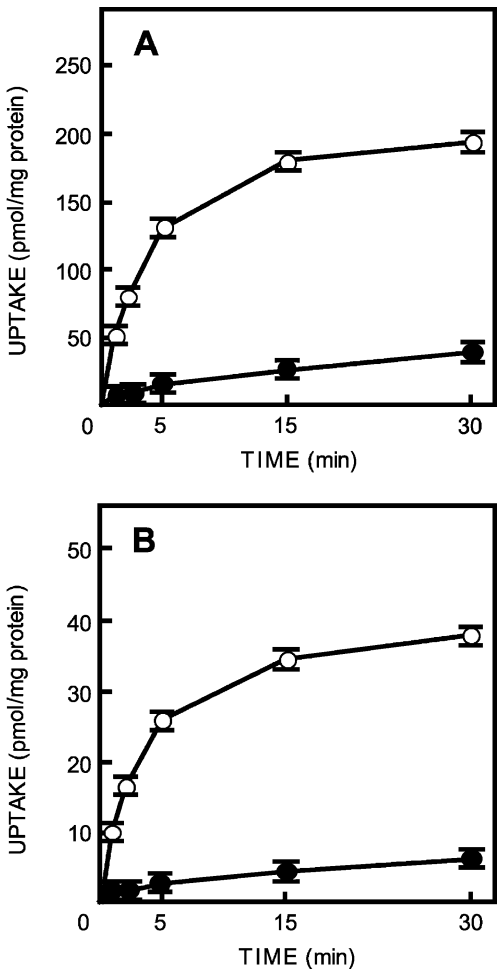

Fig. 1. Uptake of grepafloxacin (A) and levofloxacin (B) by Caco-2 cells. The monolayers were incubated for the indicated periods at 37 °C (○) or 4 °C (●) with [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM). Each point represents the mean ± S.E. of three monolayers.

3.2. Concentration-dependence of grepafloxacin and levofloxacin uptake

To characterize the uptake of grepafloxacin and levofloxacin, the concentration-dependence of the uptake by Caco-2 cell monolayers was examined. Fig. 2 shows the uptake of grepafloxacin and levofloxacin at 2 min as a function of substrate concentration. The relationship between concentration and the uptake rate approached saturation. The kinetic parameters were calculated using the following equation: $V = V_{max} S/(K_m + S) + K_d S$, where V is the transport rate (nmol/mg protein per 2 min), S is the substrate concentration in the medium (mM), K_m is the Michaelis–Menten constant (mM), V_{max} is the maximum velocity by the saturable process (nmol/mg protein per 2 min) and K_d is the coefficient of nonsaturable transport, mainly simple diffusion (nmol/mg protein per 2 min/mM). The data were fitted to the above equation by nonlinear least squares regression analysis. The apparent K_m and V_{max} values for the saturable uptake of grepafloxacin and levofloxacin were 3.9 mM and 36.1 nmol/mg protein per 2 min, and 9.3 mM and 19.1 nmol/mg protein per 2 min, respectively.

3.3. Effect of pH on uptake of grepafloxacin and levofloxacin

Next, we examined the effects of extracellular pH on grepafloxacin and levofloxacin uptake. Grepafloxacin uptake was markedly stimulated by increasing the pH of the medium (Fig. 3A). On the other hand, levofloxacin uptake was highest at weakly acidic pH (pH 6.4, Fig. 3B). Uptake of these quinolones at lowering temperature (4 °C) did not show pH-dependence (Fig. 3).

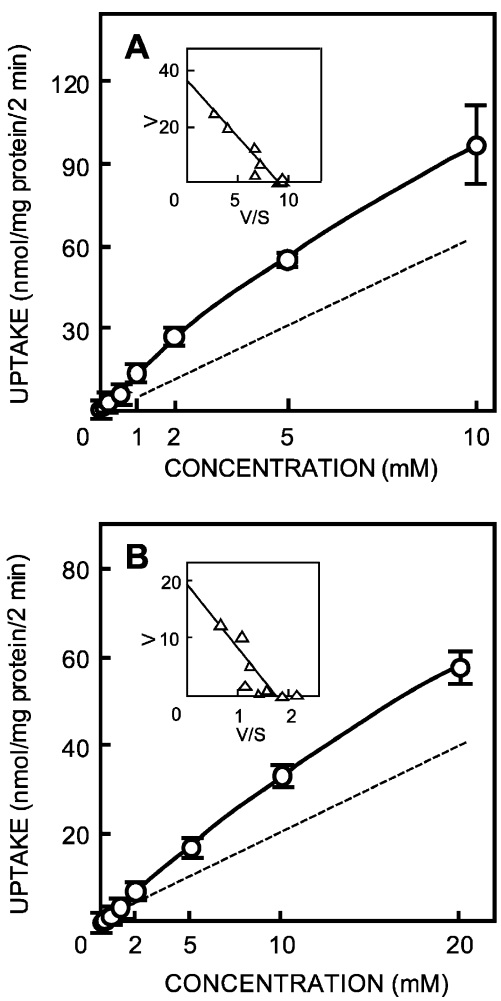

Fig. 2. Concentration-dependence of the uptake of grepafloxacin (A) and levofloxacin (B) by Caco-2 cells. Caco-2 cell monolayers were incubated at 37 °C for 2 min with varying concentrations of grepafloxacin or levofloxacin. The solid and dotted lines represent the estimated overall and nonsaturable transport, respectively. The insets show the Eadie–Hofstee plots of uptake after correction for the nonsaturable component. Each point represents the mean ± S.E. of three monolayers.

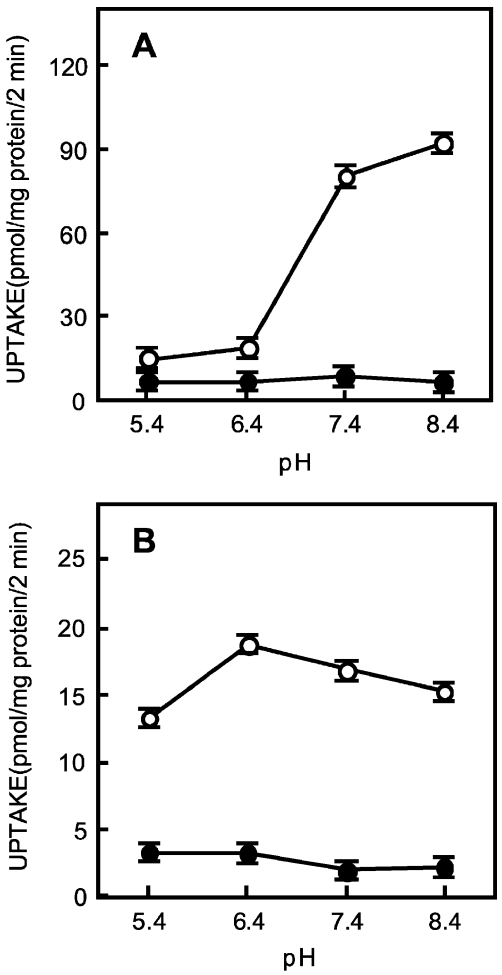

Fig. 3. Effects of pH on grepafloxacin (A) and levofloxacin (B) uptakes by Caco-2 cells. The monolayers were incubated in medium at each pH containing [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM) at 37 °C (○) or 4 °C (●). Each point represents the mean ± S.E. of three monolayers.

3.4. Effects of extracellular Na^+, Cl^- or K^+ on uptake of grepafloxacin and levofloxacin

Some transporters have been reported to show dependence on Na^+, Cl^- or membrane potential (Hediger and Rhoads, 1994; McGivan and Pastor-Anglada, 1994; Okuda et al., 1999). We next examined the effects of medium composition on the uptake of grepafloxacin and levofloxacin. As shown in Fig. 4, the uptake of grepafloxacin and levofloxacin under Cl^--free conditions was 1.2-fold greater than that of the respective control. Neither Na^+-free nor high-K^+ conditions affected the uptake of grepafloxacin or levofloxacin.

3.5. Effects of ATP depletion and cyclosporin A on the uptake of grepafloxacin and levofloxacin

The effects of ATP depletion on the uptake of grepafloxacin and levofloxacin were examined. Uptake of both quinolones was markedly increased in ATP depleted condition (up to 1.8-fold for each quinolone) (Fig. 5A,C).

To clarify the contribution of P-glycoprotein to the uptake of grepafloxacin and levofloxacin, the effects of cyclosporin A, a typical inhibitor of P-glycoprotein, were examined. However, cyclosporin A (10 μM) did not alter the uptake of grepafloxacin and levofloxacin (Fig. 5B,D).

3.6. Effects of various compounds on the uptake of grepafloxacin and levofloxacin under Cl^--free condition

To characterize the absorptive transport system of grepafloxacin and levofloxacin, inhibition experiments were performed under Cl^--free condition. Uptake of

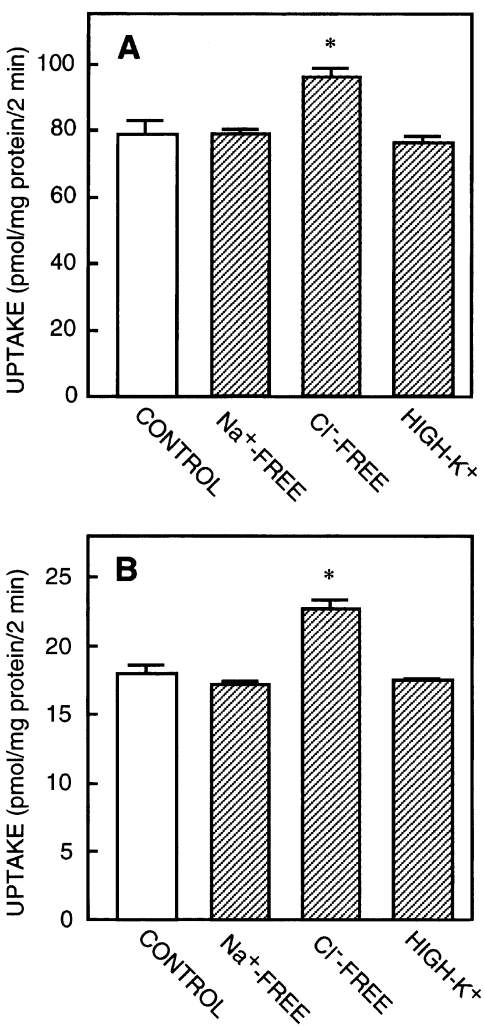

Fig. 4. Effects of extracellular Na^+, Cl^- and K^+ on the uptake of grepafloxacin (A) and levofloxacin (B) by Caco-2 cells. The monolayers were incubated at 37 °C for 2 min with Na^+-free, Cl^--free or high K^+ medium (pH 7.4) containing [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM). Each column represents the mean ± S.E. of three monolayers. * $P < 0.05$, significantly different from control.

grepafloxacin was significantly inhibited by unlabeled grepafloxacin, levofloxacin, quinidine and DIDS, and that of levofloxacin was inhibited by unlabeled levofloxacin, grepafloxacin and quinidine (Table 1). The typical organic cation transport system substrate tetraethylammonium and organic anion transport system substrate *p*-aminohippurate did not affect the uptake of these quinolones. Cimetidine, probenecid, amino acids (glycine, leucine, tryptophan, glutamate, lysine, β-alanine and GABA), monocarboxylic acids (lactate and salicylate) and β-lactam antibiotic (cephalexin) did not affect the uptake of grepafloxacin or levofloxacin (Table 1).

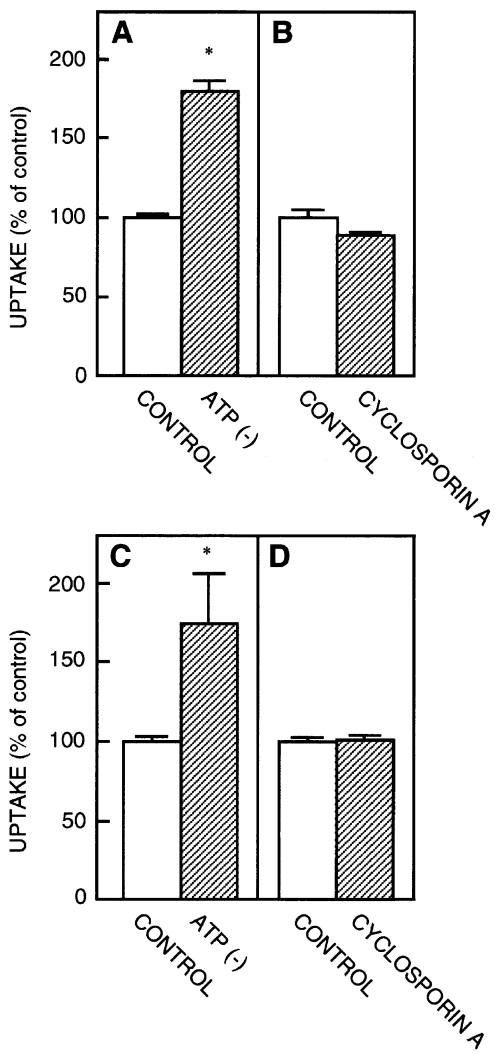

Fig. 5. Effects of ATP depletion and cyclosporin A on the uptake of grepafloxacin (A, B) and levofloxacin (C, D) by Caco-2 cells. (A, C) The monolayers were preincubated for 20 min at 37 °C with ATP depleted medium (pH 7.4). The medium was then removed, and monolayers were incubated with the same medium containing [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM) for 2 min at 37 °C. Each column represents the mean ± S.E. of six monolayers. (B, D) The monolayers were preincubated for 15 min at 37 °C in the absence or presence of 10 μM cyclosporin A (pH 7.4). After removal of the medium, cell monolayers were incubated with the same medium containing [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM) for 2 min at 37 °C. Each column represents the mean ± S.E. of three monolayers. * $P < 0.05$, significantly different from control.

Table 1
Effect of various compounds on uptake of grepafloxacin or levofloxacin under Cl^--free conditions by Caco-2 cells
The monolayers were incubated with [^{14}C]grepafloxacin (5 μM) or [^{14}C]levofloxacin (5 μM) and [^{3}H]mannitol (5 μM) for 2 min at 37 °C in the presence or absence of inhibitors. The concentrations of inhibitors were 10 mM, except for levofloxacin (20 mM), quinidine (5 mM) and DIDS (1 mM). Each value represents the mean ± S.E. of three to six monolayers.

Inhibitors	Grepafloxacin uptake (% of control)	Levofloxacin uptake (% of control)
None	100.0 ± 5.4	100.0 ± 3.3
Quinolones		
Grepafloxacin	70.8 ± 2.3 *	77.1 ± 4.6 *
Levofloxacin	78.7 ± 1.8 *	68.2 ± 3.1 *
Organic cations		
Tetraethylammonium	98.5 ± 0.9	96.2 ± 4.0
Cimetidine	107.0 ± 7.3	106.1 ± 0.7
Quinidine	51.8 ± 1.1 *	74.3 ± 2.8 *
Organic anions		
p-Aminohippurate	95.2 ± 2.7	105.0 ± 1.3
Probenecid	117.9 ± 3.9	109.4 ± 6.7
DIDS	78.0 ± 3.5 *	92.7 ± 1.9
Amino acids		
Glycine	92.7 ± 1.7	97.7 ± 4.1
Leucine	110.1 ± 2.0	109.6 ± 3.9
Tryptophan	88.1 ± 0.9	103.4 ± 5.9
Glutamate	109.5 ± 5.2	114.2 ± 10.0
Lysine	116.3 ± 5.2	101.5 ± 3.9
β-Alanine	112.6 ± 6.7	99.3 ± 6.0
GABA	111.5 ± 3.8	103.0 ± 3.4
β-Lactam antibiotic		
Cephalexin	97.6 ± 4.5	103.4 ± 1.0
Monocarboxylic acids		
Lactate	110.1 ± 1.5	94.1 ± 4.6
Salicylate	109.8 ± 1.2	97.5 ± 4.7

* $P < 0.05$, significantly different from control.

4. Discussion

We previously reported that grepafloxacin and levofloxacin were predominantly transported in the basolateral-to-apical direction in Caco-2 cells (Yamaguchi et al., 2000). However, because these quinolones are relatively well absorbed from the intestine after oral administration, we hypothesized that specific mechanisms might be involved in the absorption of these drugs. In the present study, the transport characteristics of grepafloxacin and levofloxacin were evaluated using Caco-2 cells as a model of the intestinal epithelium.

Uptake of both quinolones was rapid, temperature-dependent and saturable. Grepafloxacin uptake was strongly stimulated by increasing pH, while levofloxacin uptake was slightly stimulated at weakly acidic pH. However, these phenomena were not observed at lowering temperature. These results indicated that grepafloxacin and lev-

ofloxacin transport across the apical membrane in Caco-2 cells were carrier-mediated processes, with different pH profiles between the two substrates. We demonstrated previously that levofloxacin was a substrate for P-glycoprotein using LLC-GA5-COL150 cell monolayers which overexpressed human P-glycoprotein (Ito et al., 1997), and that gastrointestinal secretion of grepafloxacin was mainly mediated by P-glycoprotein in Caco-2 cells (Yamaguchi et al., 2000). However, because cyclosporin A showed no effect on the uptake of grepafloxacin or levofloxacin at each pH (data not shown), we considered that pH dependency was not due to P-glycoprotein-mediated transport. The pH dependence of quinolone uptake was considered to be due to alterations in their lipophilicity due to pH changes (log P at pH 5.4, 6.4, 7.4 and 8.4; −0.20, −0.23, 0.03 and 0.36 for grepafloxacin; −0.67, −0.53, −0.58 and −0.76 for levofloxacin, unpublished data). And the lipophilicity of each quinolone appeared to be related to the ratio of zwitterions in each pH (pK_{a_1} and pK_{a_2}, 7.1 and 8.8 for grepafloxacin; 5.5 and 8.0 for levofloxacin). Therefore, lipophilicity was a determinant factor of the affinity, and zwitterions might have higher affinity for the transport system.

We previously demonstrated that the basolateral-to-apical flux of quinolones was mediated by P-glycoprotein and a specific transport system (Yamaguchi et al., 2000). In this study, the uptake of grepafloxacin and levofloxacin increased under Cl^--free and ATP depleted conditions. These results indicated that the uptake of quinolones at 2 min could be evaluated including efflux transport. As grepafloxacin and levofloxacin uptakes were not affected by cyclosporin A, we considered that another efflux transport system distinct from P-glycoprotein affected the uptake of quinolones.

Yamaguchi et al. (1991) reported that sparfloxacin absorption was mediated by dipeptide transporter in the rat intestine. On the other hand, Rabbaa et al. (1997) showed that ofloxacin absorption was mediated by multiple transport systems, interacting with amino acids, distinct from dipeptide transporter or some amino acid transporters. Although both dipeptide transporter and amino acid transporter are also expressed in Caco-2 cells similar to the small intestine (Saito and Inui, 1993; McGivan and Pastor-Anglada, 1994), no significant inhibition for grepafloxacin and levofloxacin uptakes was observed by β-lactam antibiotic or neutral, basic and acidic amino acids and β-alanine and GABA. These results indicated that the uptake of grepafloxacin and levofloxacin by Caco-2 cells was not mediated by dipeptide transporter or amino acid transporters. Several hydrophilic compounds were reported to be absorbed via specialized transporters including monocarboxylic acid transporter (Tsuji and Tamai, 1996). In our study, lactate and salicylate did not affect grepafloxacin and levofloxacin uptakes, suggesting no contribution of monocarboxylic acid transporter. Grepafloxacin and levofloxacin are zwitterions at physiological pH, so we examined the effects of organic cations and anions on the uptake of these quinolones. However, no effects were observed by tetraethylammonium and cimetidine, or *p*-aminohippurate and probenecid. On the other hand, quinidine inhibited the uptake of grepafloxacin and levofloxacin, and DIDS inhibited the grepafloxacin uptake. These results suggested that grepafloxacin and levofloxacin uptakes were mediated by a novel transport system. The reason for the differences in the effect of DIDS on grepafloxacin and levofloxacin uptakes was unclear, but might have been related to the ratio of specific to overall transport of each quinolone.

Some reports indicated potential-dependent quinolone uptake using rat intestinal brush-border membrane vesicles (Iseki et al., 1992, 1998; Hirano et al., 1994, 1995), but in the present study no significant differences were observed in high-K^+ medium, suggesting that the transport system in Caco-2 cells was potential-independent. The different results might be caused by the uptake conditions between intact cells and membrane vesicles. Because the uptake of membrane vesicles is very artificial compared with that of intact cells, the contribution of membrane potential to the uptake of quinolones might be little in Caco-2 cells. Murata et al. (1999) demonstrated Na^+-dependent uptake of HSR-903 ((*S*)-(−)-5-amino-7-(7-amino-5-azaspiro[2.4]hept-5-yl)1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-4-oxoquinoline-3-carboxylic acid methanesulfonate), a newly developed quinolone, by isolated lung cells. However, the uptake of grepafloxacin and levofloxacin was Na^+-independent in Caco-2 cells. Different transport systems mediating quinolones uptake could be expressed in the lung and intestine.

In conclusion, our results suggested that a specific transport system mediates the uptake of grepafloxacin and levofloxacin across the apical membrane in Caco-2 cells. The transport system appeared to have novel characteristics different from organic cation and anion transporters, amino acid transporters and dipeptide transporter. This transport system might contribute to the high absorption rate of quinolone antibacterial drugs from the intestine.

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