Saturable Transport of H_2-Antagonists Ranitidine and Famotidine Across Caco-2 Cell Monolayers

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Abstract □ The purpose of this study was to investigate the mechanism by which the H_2-antagonists ranitidine and famotidine interacted with the paracellular space during their transport across Caco-2 cell monolayers. Transport experiments with ranitidine and famotidine across Caco-2 cell monolayers were performed to determine the apical-to-basolateral flux at various concentrations. Kinetic analysis of the transport data showed that ranitidine and famotidine were transported by both saturable and nonsaturable processes. Na^+,K^+-ATPase inhibitor ouabain and metabolic inhibitors sodium azide + 2-deoxy-D-glucose did not affect ranitidine transport, suggesting that the active transport was not involved. Famotidine and some other guanidine-containing compounds, e.g., guanethidine, Arg-Gly, L-arginine methyl ester, and L-argininamide, inhibited the transport of ranitidine, whereas other guanidine-containing compounds with an additional negative charge, e.g., L-arginine, did not. 2,4,6-Triaminopyrimidine (TAP), an inhibitor of paracelluar cationic conductance, also inhibited the transport of both ranitidine and famotidine. On the basis of these results, it is proposed that the saturable transport of ranitidine and famotidine across Caco-2 cell monolayers appears to be via a facilitated diffusion process mediated by the paracellular anionic sites. This mechanism is consistent with the observation that ranitidine and famotidine caused a concentration-dependent increase in transepithelial electrical resistance (TEER) across Caco-2 cell monolayers, presumably by blocking the paracellular anionic sites and thus inhibiting the flux of cations (e.g., Na^+).

Introduction

Intestinal epithelium presents a major barrier to orally administered drugs.[1,2] The drug molecules have to traverse this barrier by entering the epithelial cells from the apical (or luminal) side and exiting from the basolateral (or serosal) side (i.e., the transcellular transport), or by passing through the intercellular space (i.e., the paracellular transport). The transcellular transport occurs either via a passive diffusion process or via a carrier-mediated process, involving one of many carrier proteins (i.e., carrier for amino acids, di/tri peptides, glucose, bile acids, etc.).[3] The paracellular transport, involving passive diffusion of compounds in aqueous solution, is less efficient than the transcellular process because of the much lower surface area available to the compounds entering the intercellular space. In addition, presence of the highly specialized structure in the intercellular space restricts the free passage of compounds traversing the intestinal epithelium via the paracellular process.[4−7] Hence, only hydrophilic compounds of low molecular weight (e.g., mannitol) cross the intestinal epithelium predominantly via the paracellular route.

The H_2-antagonist ranitidine appears to be absorbed predominantly via the paracellular pathway based on the studies with Caco-2 cell monolayers as in vitro model of intestinal mucosa.[8] This conclusion was based on the observation that the permeability coefficient (P_{app}) of ranitidine across Caco-2 cell monolayers increased by 15−20-fold when Ca^{2+} was removed from the transport medium, thereby compromising the integrity of the tight junctions.[8] This was further confirmed by the observation that the uptake of ranitidine into Caco-2 cells was minimal.[8] The paracellular transport of ranitidine is consistent with the report that absorption of ranitidine in humans after oral administration is incomplete and that its bioavailability is ∼50% despite little or no first pass metabolism.[9] During the investigation of the mechanism of transport of ranitidine it was uncovered that H_2-antagonists—ranitidine, famotidine, cimetidine, and nizatidine—appeared to affect the tight junctions in Caco-2 cell monolayers as evidenced by a concentration-dependent increase in the transepithelial electrical resistance (TEER) across Caco-2 cell monolayers,[10] which is an indicator of paracellular ionic permeability.[11−13] The increase in TEER was accompanied by a decrease in their own permeability across Caco-2 cell monolayers.[10] These results suggest that H_2-antagonists may affect their own absorption by a mechanism that is related to the mechanism by which they cause an increase in TEER. An inverse relationship between the ability of the four H_2-antagonists to cause an increase in TEER across Caco-2 cell monolayers and their human bioavailability[9,10,14] provides support to this hypothesis.

In light of these observations, we have investigated the mechanism by which the H_2-antagonists cause an increase in TEER and affect their own transport across Caco-2 cell monolayers. Our results suggest that H_2-antagonists cause an increase in TEER by binding to the anionic centers in the paracellular space and thereby decreasing the cationic conductance across the cell monolayers. During our investigation, we have found that ranitidine and famotidine traverse the Caco-2 cell monolayers by a combination of saturable and nonsaturable mechanisms. In this report we have characterized the saturable transport of ranitidine and famotidine and proposed a mechanism of transport for these compounds that is consistent with the saturable transport kinetics as well as their ability to cause an increase in TEER across Caco-2 cell monolayers.

Materials and Methods

Materials—Eagle's minimum essential medium (with Earle's salts and L-glutamate), fetal bovine serum (FBS), nonessential amino acids (NEAA, ×100), and 0.05% trypsin-EDTA solution were obtained from Gibco Laboratories, Grand Island, NY. Hank's balanced salt solution (HBSS, ×1), ranitidine hydrochloride,

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famotidine, 2,4,6-triaminopyrimidine (TAP), guanethidine monosulfate, Arg-Gly hydrochloride, Gly-Gly, L-arginine hydrochloride, L-argininamide dihydrochloride, L-arginine methyl ester dihydrochloride, glycylsarcosine (Gly-Sar), mannitol, ouabain, sodium azide, 2-deoxy-D-glucose, 2,4-dinitrophenol, antibiotic antimycotic solution (×100), 2-(*N*-morpholino)ethanesulfonic acid (MES), and D-(+)-glucose were purchased from Sigma Chemical Co., St. Louis, MO. *N*-Hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES, 1 M) was purchased from Lineberger Comprehensive Cancer Center, the University of North Carolina, Chapel Hill, NC. Lucifer yellow CH was purchased from Molecular Probes, Eugene, OR. [^{14}C]-Mannitol (51.5 mCi/mmol) and [^{14}C]PEG-4000 (11.0 mCi/g) were obtained from NEN Research Products, Boston, MA. [^{3}H]Gly-Sar (30 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc., St. Louis, MO.

Cell Culture—The Caco-2 cell line was obtained from Glaxo-Wellcome, Inc., Research Triangle Park, NC, and cultured as described previously.[10] Briefly, Caco-2 cells were cultured at 37 °C in minimum essential medium, supplemented with 10% FBS, 1% NEAA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B in an atmosphere of 5% CO_2 and 90% relative humidity. The cells were passaged every 4−6 days at a split ratio of 1:20 at about 90% confluency, using trypsin−EDTA. Caco-2 cells (passage 50∼55) were seeded at a density of 60 000 cells/cm^2 on polycarbonate membranes of Transwells (12 mm i.d., 3.0 μm pore size, Costar, Cambridge, MA). Medium was changed the day after seeding and every other day thereafter (apical volume 0.5 mL, basolateral volume 1.5 mL). The cell monolayers were used 20−25 days postseeding. TEER was measured as described later. TEER and apical-to-basolateral flux for [^{14}C]mannitol, a paracellular transport marker, were used to ensure cell monolayer integrity. Monolayers having TEER values above 300 Ω·cm^2 and the mannitol flux < 0.5%/h were used in the studies.

Measurement of TEER—EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, Sarasota, FL) and Endohm-12 electrode (World Precision Instruments, Sarasota, FL) were used to measure TEER across the Caco-2 cell monolayers.[10] The cell monolayers were preincubated for 1 h at 37 °C with transport buffer (HBSS supplemented with 25 mM D-glucose and 10 mM HEPES, pH 7.2), and TEER was measured. The experiments were initiated by replacing the apical buffer with the compound of interest dissolved in an appropriate transport buffer (HBSS supplemented with 25 mM D-glucose and 10 mM HEPES, pH 7.2 or HBSS supplemented with 25 mM D-glucose and 10 mM MES, pH 6.0). The cell monolayers were incubated at 37 °C, and TEER was measured at selected times. All the experiments were performed in triplicate.

Transport Studies—Transport experiments were performed as described previously.[10] Briefly, cell monolayers were incubated for 1 h at 37 °C with the transport buffer (pH 7.2), and TEER was measured. Transport experiments were initiated by replacing the apical buffer with 0.4 mL of the transport buffer (pH 7.2 or 6.0) containing the compound being investigated. The inserts were transferred at selected times to a 12-well cell culture cluster (Costar, Cambridge, MA) containing fresh transport buffer (pH 7.2). The temperature was maintained at 37 °C during the transport experiments. TEER was measured after the experiments. No treatment caused a significant decrease in TEER compared to the control monolayers. The amount of drugs in the receiver side was determined as a function of time (see Figure 3A) to calculate the flux (J) across the cell monolayers. All transport experiments were carried out under sink conditions as the concentrations of drugs in the basolateral side remained at least 100-fold lower than those in the apical side. The amount of radiolabeled compounds was measured by using liquid scintillation counter (Tri-Carb 4000, Packard, Downers Grove, IL). The amount of ranitidine or famotidine was quantified by HPLC (1100 series, Hewlett-Packard, Waldbronn, Germany) using an ODS-AQ C18 column (250 × 4.6 (i.d.) mm, YMC Inc., Wilmington, NC) of 5 μm packing and 120 Å pore size and an isocratic mobile phase (65% 50 mM phosphate buffer, pH 6.0 and 35% methanol for ranitidine, and 80% 50 mM phosphate buffer, pH 6.0 and 20% methanol for famotidine).[8,10] The flow rate was 1.0 mL/min. Ranitidine and famotidine were detected by UV at 320 and 280 nm, respectively. Under these conditions, the retention times for ranitidine and famotidine were 5.6 and 9.6 min, respectively, and no other peaks were detected after the transport experiments.

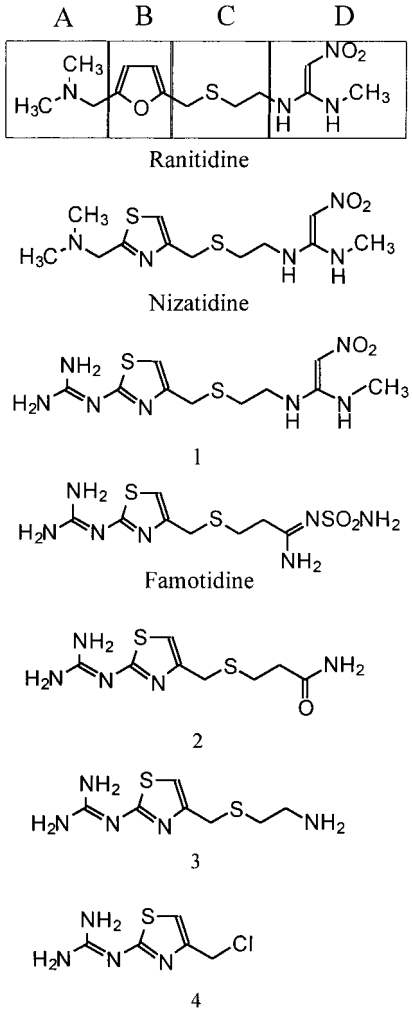

Figure 1—Structures of H_2-antagonists and their derivatives.

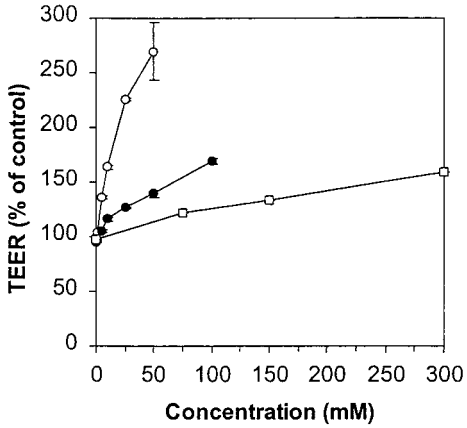

Figure 2—Effect of ranitidine and famotidine on TEER across Caco-2 cell monolayers. Effect of mannitol on TEER was evaluated to demonstrate that the effect of ranitidine and famotidine was selective and not just due to the change in osmolality. TEER was measured 15 min after incubating the cell monolayers with various concentrations of drugs on the apical side (pH 7.2). Percent change of the TEER values was calculated relative to the value at time zero (303 ∼ 419 Ω·cm^2). ○, Famotidine; ●, ranitidine; □, mannitol. Values are the mean of three measurements ± sd. The pH of the famotidine solutions above 10 mM was 6.5 (instead of 7.2); however, the change in pH alone did not contribute to the increase in TEER.

Inhibition of Active Transport—For all the studies described in this section, cell monolayers were preincubated for 30 min at 37 °C with the transport buffer (pH 7.2) and TEER was measured. For the inhibition of Na^+,K^+-ATPase,[15] cell monolayers were

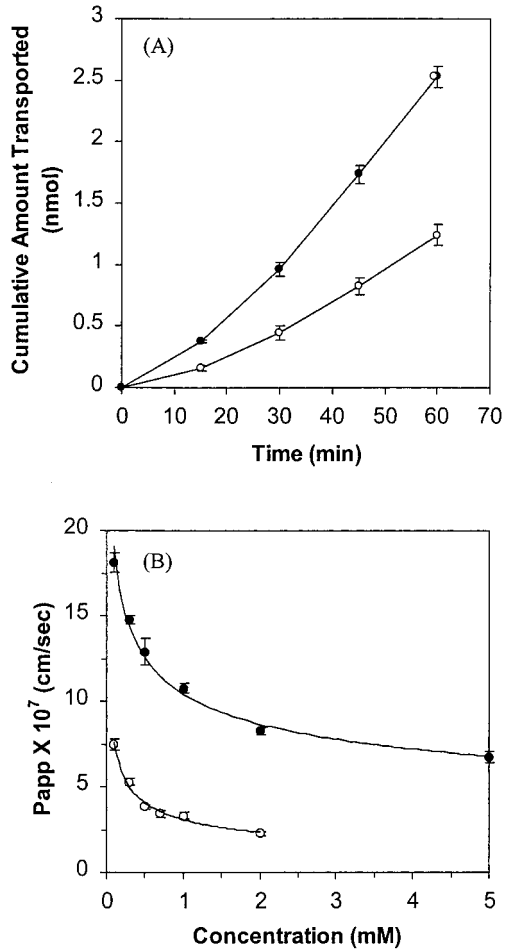

Figure 3—(A) Time course of apical-to-basolateral transport of ranitidine (0.5 mM) and famotidine (0.5 mM) across Caco-2 cell monolayers; (B) permeability of ranitidine and famotidine across Caco-2 cell monolayers as a function of concentration. P_{app} values were calculated from the apical-to-basolateral flux (J) determined in a linear transport region (30–60 min) at various concentrations. The pH of both apical and basolateral sides was 7.2. ●, Ranitidine; ○, Famotidine. Values are the mean of three measurements ± sd.

further incubated for 30 min at 37 °C with ouabain (5 mM) dissolved in the transport buffer (pH 7.2) on both apical and basolateral sides. Ranitidine (0.1 mM) in the transport buffer (pH 7.2) containing ouabain (5 mM) was then added to the apical side. For metabolic inhibition,[15] cell monolayers were incubated for 30 min at 37 °C with sodium azide (1 mM) and 2-deoxy-D-glucose (50 mM), or 2,4-dinitrophenol (1 mM), dissolved in the transport buffer (pH 7.2) on both apical and basolateral sides. Ranitidine (0.1 mM) in the transport buffer (pH 7.2) containing metabolic inhibitors was then added to the apical side. The transport experiments were conducted for 1 h. For control studies, a mixture of [^{3}H]Gly-Sar (0.1 mM, 0.5 μCi/ml) and [^{14}C]mannitol (0.1 mM, 1.0 μCi/ml) in the transport buffer (pH 6.0) was applied to the apical side after incubating the cell monolayers with active transport inhibitors.[16] The pH of the apical solution was adjusted to 6.0 for the control experiments since Gly-Sar (positive control) is a substrate of H^+-coupled dipeptide transporter.[16]

Data Analysis—Apparent permeability coefficients (P_{app}, cm/s) were calculated using eq 1.[10]

$$P_{app} = \frac{1}{AC_o}\frac{dQ}{dt} \quad (1)$$

where dQ/dt is the flux (J) across the monolayer (nmol/s) determined experimentally by measuring the amount of the compounds transported as a function of time (e.g., see Figure 3A for measurement of ranitidine and famotidine flux), A is the surface area of the porous membrane (cm^2), and C_o is the initial concentration (nmol/mL) in the donor side. Kinetic parameters for the transport

Table 1—Effect of Famotidine Derivatives on TEER across Caco-2 Cell Monolayers

compound[a]	TEER[b] (% of control)
1	217 ± 7
2	201 ± 1
3	158 ± 17
4	235 ± 11
ranitidine	127 ± 0.3
nizatidine	117 ± 3[c]
famotidine	193 ± 22

[a] The concentration of nizatidine was 50 mM while others were 25 mM. [b] Measured 15 min after incubating the cell monolayers with a compound in the apical side. Percent change of the TEER values was calculated relative to the value at time zero (366–442 Ω·cm^2). Values are the mean of three measurements ± sd. [c] Taken from ref 10.

of ranitidine and famotidine were calculated by fitting the data to eq 2 using nonlinear regression analysis (WINNONLIN 1.1, Scientific Consulting Inc., Apex, NC):[17,18]

$$J' = \frac{J'_{max}S}{K_m(app) + S} + K_dS \quad (2)$$

where J' (pmol·min^{-1}·cm^{-2}) is the flux (J) normalized to unit surface area, K_m(app) (mM) is a constant equivalent to a Michaelis–Menten constant, J'_{max} is the maximal flux for the saturable term (pmol·min^{-1}·cm^{-2}), K_d is the constant for the nonsaturable term (nL·min^{-1}·cm^{-2}), and S is the concentration in the donor side (mM). The statistical significance of differences between treatments was evaluated using Student's t tests, with a significance level of $p < 0.05$.

Synthesis of Famotidine Derivatives—Famotidine derivatives used in this study (cf. Figure 1) were prepared as described in the literature.[19,20]

Results

Effect of H_2-Antagonists on TEER—Ranitidine and famotidine caused a concentration-dependent but saturable increase in TEER when applied to the apical side of Caco-2 monolayers (Figure 2), as reported previously.[10] A much smaller increase was observed when the monolayers were treated with mannitol solutions of similar osmolality (Figure 2). These results indicated that the increase in TEER caused by ranitidine and famotidine did not simply result from the hyperosmolality of the drug solutions. The specificity of this effect was further evidenced by the fact that famotidine was much more potent than ranitidine in causing the TEER increase across Caco-2 cell monolayers (Figure 2).

Structural Requirements for the Effect of H_2-antagonists on TEER—A systematic study was undertaken to define the structural requirements for the effect of H_2-antagonists and related compounds on TEER. The role of the four key moieties in the H_2-antagonists (Figure 1) in causing an increase in TEER was investigated by comparison of the potencies of appropriate pairs of compounds (Table 1). The heteroaromatic ring (part B) does not seem to play a significant role, as indicated by the marginal difference in the potency between ranitidine and nizatidine (see Figure 1 and Table 1 for all comparisons). The thioether linker (part C) is not a primary determinant of the effect since the potency varies dramatically among compounds which share this moiety. Substitution of the *N*-sulfamoylamidine moiety of famotidine by 1-nitro-2-(methylamino)-2-aminoethylene group (compound **1**) caused no significant change in the potency, indicating that part D is also not a primary determinant of the activity. This was confirmed by the observation that the potency was maintained when part D was replaced with various func-

Table 2—Effect of Famotidine on the Transport of Paracellular Markers

Paracellular Markers	net charge	mol wt	% of control[a]
[^{14}C]mannitol (100 μM, 20 μCi/μmol)	0	182	114 ± 9
[^{14}C]PEG-4000 (100 μM, 5 μCi/μmol)	0	4000	97 ± 2
lucifer yellow CH (250 μM)	–2	457	79 ± 13
ranitidine (100 μM)	+1	314	22 ± 0.2[b]

[a] Determined by measuring the apical-to-basolateral transport of compounds for 1 h across Caco-2 cell monolayers in the absence (control) or presence of famotidine (5 mM) on the apical side (pH 7.2). Values are the mean of three measurements ± sd. [b] $p < 0.05$ compared to control.

tional groups such as amide (compound **2**), amine (compound **3**). Similarly, potency was maintained when part (C + D) was replaced with a chloromethyl group (compound **4**). The basic side chain represented in part A appears to be the essential moiety for the effect since a dramatic increase in the potency was observed when the dimethylaminomethyl group of nizatidine was substituted with guanidine group (compound **1**). This observation also indicates that the potent effect of famotidine may be due to the presence of the guanidine group.

Effect of Famotidine on the Transport of Paracellular Markers—The effect of famotidine on paracellular permeability was examined by measuring the permeability (P_{app}) of various paracellular markers across Caco-2 cell monolayers in the presence of famotidine (Table 2). This was done to determine if the increase in TEER by H_2-antagonists was caused by tightening of the paracellular space. Famotidine did not cause a significant decrease in the permeability of neutral ([^{14}C]mannitol, and [^{14}C]PEG-4000) or anionic (Lucifer Yellow) paracellular markers. In contrast, famotidine inhibited the transport of ranitidine, a cationic compound that is transported via the paracellular route.[8] These results suggest that the increase in TEER caused by famotidine (and other H_2-antagonists) is not due to tightening of the tight junctions in the paracellular space; rather, famotidine appears to inhibit the transport of only positively charged entities through the paracellular space. Thus, the increase in TEER caused by famotidine (and other H_2-antagonists) is likely to be due to inhibition of the cation-selective ionic conductance, i.e., Na^+ transport, mediated by the anionic centers in the paracellular space.[21–23] This mechanism is consistent with our earlier finding that guanidine group, which is positively charged at neutral pH, is essential for causing an increase in TEER across Caco-2 cells by famotidine and its derivatives (Table 1).

Transport of Ranitidine and Famotidine—Consistent with above observations, the apical-to-basolateral permeability of both ranitidine and famotidine through Caco-2 cell monolayers was found to be dependent on concentration (Figure 3B). The P_{app} values decreased exponentially with concentration, but appeared to reach plateau above 2 mM for ranitidine and 0.7 mM for famotidine (Figure 3B). The decrease in P_{app} as a function of concentration suggests that both ranitidine and famotidine inhibit their own transport (Figure 3B). These results also indicate that ranitidine and famotidine are transported by a process other than simple passive diffusion, because the P_{app} values should remain constant over the entire concentration range (under sink condition) if passive diffusion is the major transport mechanism. We have previously reported a concentration-dependent decrease in P_{app} values for ranitidine and famotidine;[10] however, those studies were conducted at concentrations ≥ 5 mM. At these high

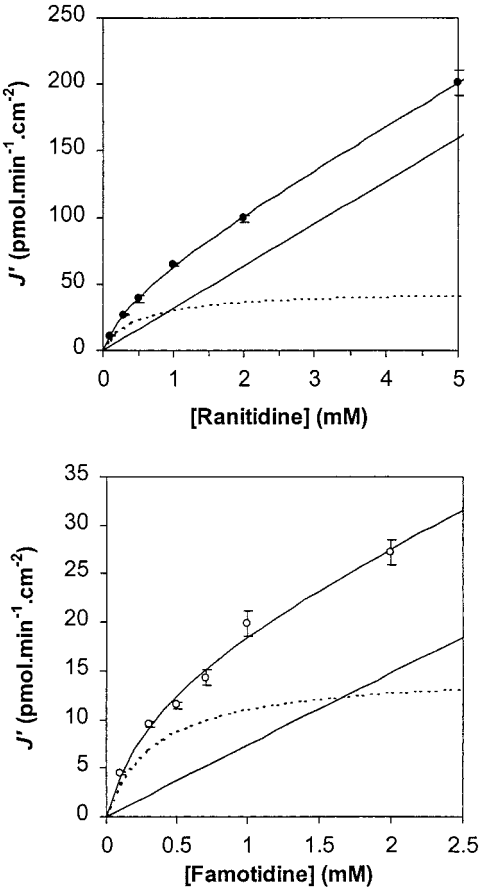

Figure 4—Transport of ranitidine and famotidine across Caco-2 cell monolayers as a function of concentration. Apical-to-basolateral flux (J') (same data used in Figure 3) was plotted as a function of concentration. The straight lines represent the calculated nonsaturable transport and the dotted curves represent the calculated saturable transport (see eq 2). ●, Ranitidine; ○, Famotidine. Values are the mean of three measurements ± sd.

concentrations, the decrease in P_{app} could be due to multiple effects, i.e., saturation of the anionic sites, osmotic effects, and a decrease in pH (famotidine only) necessary to keep the drug in solution. To obtain a better insight into the transport process for these compounds, their flux (J') was plotted as a function of concentration on the donor side (Figure 4). Under sink conditions (as is the case here, see Materials and Methods and Figure 3A), the flux (J') should increase linearly as a function of concentration in the donor side if the compounds are transported by the passive diffusion process. The flux (J') of ranitidine and famotidine increased nonlinearly with concentration (hyperbolic relationship) at relatively low concentrations (<1 mM) and then increased linearly at higher concentration. These results suggested that the apical-to-basolateral transport of ranitidine and famotidine was mediated by both saturable and nonsaturable processes.[17,18] This was also indicated by an excellent fit of the data to the curves obtained from nonlinear regression analysis with an equation consisting of both saturable and nonsaturable terms (Figure 4 and eq 2).[17,18] The calculated equivalent to Michaelis–Menten constant (K_m(app)) and the maximal flux for the saturable process (J'_{max}) for ranitidine were 0.48 mM and 45.6 pmol·min^{-1}·cm^{-2}. The corresponding values for famotidine were 0.36 mM and 15.0 pmol·min^{-1}·cm^{-2}, respectively.

Effect of Inhibitors of Active Transport on the Transport of Ranitidine—Gan et al.[8] have shown that ranitidine is transported predominantly via the paracellular pathway across Caco-2 cell monolayers. This was

Table 3—Effect of Active Transport Inhibitors on the Transport of Ranitidine

inhibitor (mM)	amount transported (%)[a]		
	ranitidine	[^{3}H]Gly-Sar	[^{14}C]mannitol
control	2.60 ± 0.08	4.52 ± 0.25	0.23 ± 0.01
ouabain (5)	2.68 ± 0.05	2.55 ± 0.11[b]	0.28 ± 0.07
sodium azide (1) + 2-deoxy-D-glucose (50)	2.49 ± 0.21	2.91 ± 0.11[b]	0.25 ± 0.002
2,4-dinitrophenol (1)	1.40 ± 0.06[b]	0.76 ± 0.16[b]	0.32 ± 0.10

[a] Determined by measuring the apical-to-basolateral transport across Caco-2 cell monolayers for 1 h in the absence (control) or presence of active transport inhibitors. The monolayers were first incubated with an inhibitor for 30 min, and ranitidine (0.1 mM) or control compound (0.1 mM) was added to the apical side to initiate the experiment (see Materials and Methods for details). [b] $p < 0.05$ compared to control. Values are the mean of three measurements ± sd.

evidenced by a dramatic increase in ranitidine transport by Ca^{2+} depletion, and by relatively low cellular uptake of ranitidine compared to that of lipophilic compounds such as testosterone and ondansetron.[8] Therefore, it is unlikely that transcellular saturable mechanisms are significantly involved in the transport of ranitidine.

It is difficult to conceive an active transport process to explain the saturable transport of ranitidine and famotidine in the absence of a significant transcellular component associated with the transport of these compounds across Caco-2 cell monolayers. However, the transport of ranitidine across Caco-2 cell monolayers was examined in the presence of Na^+,K^+-ATPase inhibitor ouabain, or metabolic inhibitors such as sodium azide + 2-deoxy-D-glucose, and 2,4-dinitrophenol, to determine if the saturable component of the transport is an active transport process.[3,15,17] In our control experiments, the active transport inhibitors significantly inhibited the apical-to-basolateral transport of [^{3}H]Gly-Sar (positive control)[16] without affecting that of [^{14}C]mannitol (negative control) at concentrations that were reported to be inhibitory to active transport processes (Table 3).[3,15,17] Ouabain and sodium azide (+ 2-deoxy-D-glucose), however, did not affect the apical-to-basolateral transport of ranitidine across Caco-2 cell monolayers (Table 3), suggesting that ranitidine is not a substrate for active transport systems (e.g. amino acids, di/tri peptides, glucose, bile acids, etc.) in Caco-2 cells. Interestingly, 2,4-dinitrophenol significantly decreased the transport of ranitidine (Table 3). The inhibitory effect of 2,4-dinitrophenol on the transport of ranitidine may argue for an active transport mechanism; however, there is no precedent for an active transport process that is inhibited by 2,4-dinitrophenol, but is insensitive to sodium azide or ouabain.

Inhibition of Ranitidine Transport by Guanidine Derivatives—To determine if the saturable transport of ranitidine and famotidine occurred via a common mechanism, the permeability (P_{app}) of ranitidine was measured in the presence of famotidine. Indeed the apical-to-basolateral transport of ranitidine (0.5 mM) was significantly inhibited by famotidine (10 mM) (Figure 5). Interestingly, other guanidine-containing compounds such as guanethidine, L-arginine methyl ester, and L-argininamide also significantly inhibited the transport of ranitidine (Figure 5). Arg-Gly, a guanidine-containing dipeptide, significantly inhibited ranitidine transport whereas Gly-Gly, a non-guanidine-containing dipeptide had little effect (Figure 5). The common structural feature shared by all these compounds that inhibited the transport of ranitidine across Caco-2 cell monolayers is the presence of a cationic center. It has been shown that the cell surface, including that making up the paracellular space, has net negative charge.[5–7] These results suggest that the saturable transport of ranitidine and famotidine may involve binding to the anionic cellular components via their basic side chains (dimethylamino group or guanidine group). Taking the predominantly paracellular transport of ranitidine[8] into consideration, it seems likely that the saturable transport of ranitidine and famotidine is mediated by the anionic sites in the paracellular space. This mechanism is consistent with our proposed mechanism for the TEER increase by ranitidine and famotidine. Interestingly, L-arginine did not cause a significant effect on the transport of ranitidine (Figure 5). However, L-arginine methyl ester and L-argininamide (in which the carboxylate group is blocked) effectively inhibited ranitidine transport, suggesting that the negative charge of the carboxylate group in L-arginine interferes with its ability to bind to the anionic sites in the paracellular space. The fact that structural components other than the cationic groups are involved in the interactions of these compounds with the anionic sites is also evident from the observation that tetramethylammonium ion does not inhibit the transport of ranitidine across Caco-2 cells (data not shown).

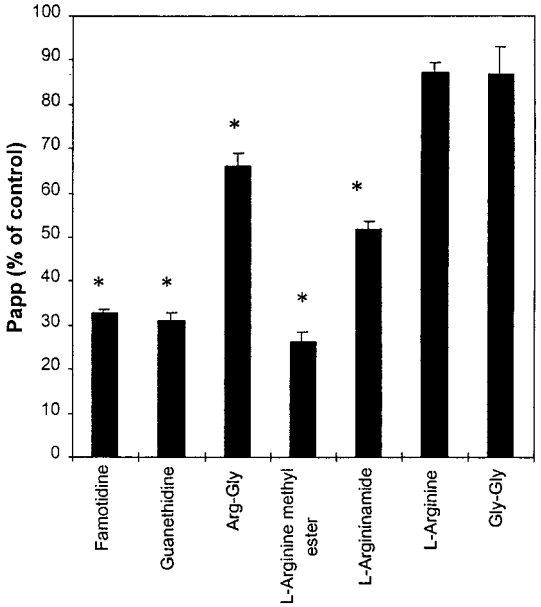

Figure 5—Effect of famotidine and other guanidine-containing compounds on the transport of ranitidine across Caco-2 cell monolayers. P_{app} values were calculated from the apical-to-basolateral flux (J) of ranitidine (0.5 mM) determined in the linear transport region (30–60 min) in the presence or absence of a guanidine-containing compound (10 mM) on the apical side (pH 7.2). The control P_{app} value was $(1.1 \pm 0.04) \times 10^{-6}$ cm/s. Values are the mean of three measurements ± sd. *, $p < 0.05$ compared to control.

Discussion

We have recently reported that ranitidine and other H_2-antagonists cause an increase in TEER across Caco-2 cell monolayers.[10] In the present study we have further characterized this effect and showed that the increase in TEER does not cause any decrease in the permeability of paracellularly transported compounds that are either neutral of anionic in nature. In contrast, paracellular transport of cationic compounds (including their own transport) is inhibited by ranitidine and famotidine. Thus, the effect of ranitidine and famotidine on TEER is clearly not due to tightening of the tight junctions as is the case for protamine.[24] We propose that H_2-antagonists cause an increase in TEER by binding to the anionic sites such as carboxylate and phosphate groups[5–7] on the cell surface in the paracellular space and inhibiting cation-selective ionic conduc-

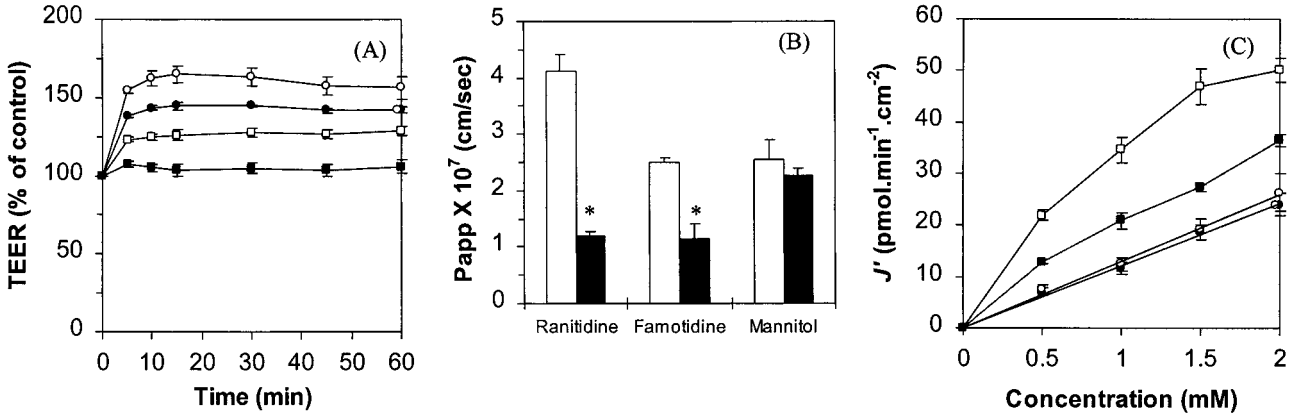

Figure 6—Effect of TAP on TEER and the transport of ranitidine and famotidine across Caco-2 cell monolayers. (A) TEER was measured at selected times after initiating the incubation of the cell monolayers with various concentrations of TAP on the apical side. Percent change of the TEER values was calculated relative to the value at time zero (370–417 $\Omega \cdot cm^2$). ■, Control; □, 5 mM; ●,10 mM; ○, 20 mM TAP. (B) P_{app} values for ranitidine, famotidine (both 1 mM) and [^{14}C]mannitol (1 mM; 2.0 μCi/ml) were calculated from the apical-to-basolateral flux (J) determined in a linear transport region (30–60 min) in the absence or presence of TAP (10 mM) on the apical side; open bars, control (– TAP); solid bars, + TAP (10 mM). (C) ●, Ranitidine and ○, famotidine flux determined in the presence of 10 mM TAP; □, ranitidine and ■, famotidine flux determined in the absence of TAP. All experiments were done at apical pH 6.0/basolaterlal pH 7.2 because TAP (pK_a = 6.72) caused an increase in TEER only in the protonated form.[21] Values are the mean of three measurements ± sd. *, $p < 0.05$ compared to control.

tance,[21–23] and not by tightening of the tight junctions as was previously suspected.[10] Such interactions between cationic compounds and paracellular anionic functionality are not without precedent. 2,4,6-Triaminopyrimidine (TAP) has been shown to inhibit paracellular cation permeation across frog gall bladder and several other epithelia with concomitant increase in TEER.[21–23] In our study, TAP caused a concentration-dependent increase in TEER across Caco-2 cell monolayers (Figure 6A). TAP also caused a significant decrease in the permeability of both ranitidine and famotidine across Caco-2 monolayers without affecting that of [^{14}C]mannitol (Figure 6B). Partial inhibition of the transport of ranitidine (1 mM) and famotidine (1 mM) by TAP is consistent with the fact that at these concentrations both the compounds traverse the cell monolayers via a combination of saturable and nonsaturable mechanisms.

The investigation of the effect of H_2-antagonists on TEER has led to the hypothesis that these compounds not only interact with anionic sites on the cell surface in the paracellular space, but that this interaction may contribute to a saturable transport mechanism. This saturable mechanism is characterized by the hyperbolic relationship between flux and the concentration in the donor side. As expected, certain cationic compounds (e.g., TAP) inhibit the transport of ranitidine and famotidine (cf., Figure 6B). In fact, in the presence of TAP the saturable component of the paracellular transport of ranitidine and famotidine is completely inhibited, such that the flux is linear with concentrations (Figure 6C).

Because TAP can also block Na^+ channels,[25] it can be argued that the increase in TEER caused by TAP as well as by ranitidine and famotidine is due to their effect on these Na^+ channels on the apical surface of Caco-2 cells. However, inhibition of ranitidine and famotidine transport, coupled with virtual elimination of the saturable component of their transport by TAP cannot be explained by its effect on the Na^+ channels because these compounds are not likely to be transported across the cell membrane through these channels.

The saturable transport of ranitidine and famotidine does not appear to be an active transport process as it is unaffected by known active transport inhibitors, i.e., Na^+,K^+-ATPase inhibitor ouabain and metabolic inhibitor sodium azide (+ 2-deoxy-D-glucose). However, the active transport cannot be completely ruled out as a possible mechanism for the saturable transport of ranitidine and famotidine, since ranitidine transport was significantly inhibited by a different metabolic inhibitor 2,4-dinitrophenol. The reason for this is unknown at this stage.

It is clear that some of the well-accepted approaches to evaluate the involvement of an active transport process (e.g., metabolic inhibition) cannot provide clearly interpretable results regarding the involvement of an active transport process for the transport of ranitidine and famotidine. However, if we take into consideration (i) the evidence for predominantly paracellular transport of ranitidine,[8] and (ii) the lack of any effect of some inhibitors of active transport (Table 3), then it is reasonable to conclude that an active transport is not likely to be involved in the translocation of ranitidine or famotidine across Caco-2 cell monolayers.

We propose a mechanism for the transport of H_2-antagonists, ranitidine and famotidine, across Caco-2 cell monolayers to explain the increase in TEER[10] caused by these compounds as well as their saturable (plus nonsaturable) transport kinetics (Figure 7). As depicted in Figure 7, ranitidine and famotidine can interact with anionic sites present on the cell surface in the paracellular space,[5–7] and thus compete with Na^+ ions (and other cations) for these binding sites resulting in decreased ionic transport and increased TEER across the cell monolayers. A similar mechanism has been proposed to explain an increase in TEER caused by the cationic hydrophilic compound TAP across frog gall bladder and several other epithelia.[21,22] The fact that ranitidine affects TEER only when it is applied from the apical side[10] of the Caco-2 cell monolayers suggests that the anionic sites are asymmetrically disposed in the paracellular space, either in the junctional complexes or to the apical side of the junctional complexes. It is conceivable that the effect of ranitidine and famotidine on TEER is due to a mechanism unrelated to their proposed binding to the paracellular anionic sites, i.e., via blocking of the Na^+ channels on the apical surface of the cells. Extensive electrophysiological studies will be necessary to determine the relative contribution of the two mechanisms in causing an increase in TEER.

As shown in Figure 7, these interactions of ranitidine (and famotidine) with anionic sites can also explain how the translocation of these compounds through the paracellular space can occur by a combination of saturable and nonsaturable processes. Their interaction with the anionic sites can assist in their translocation across the cell

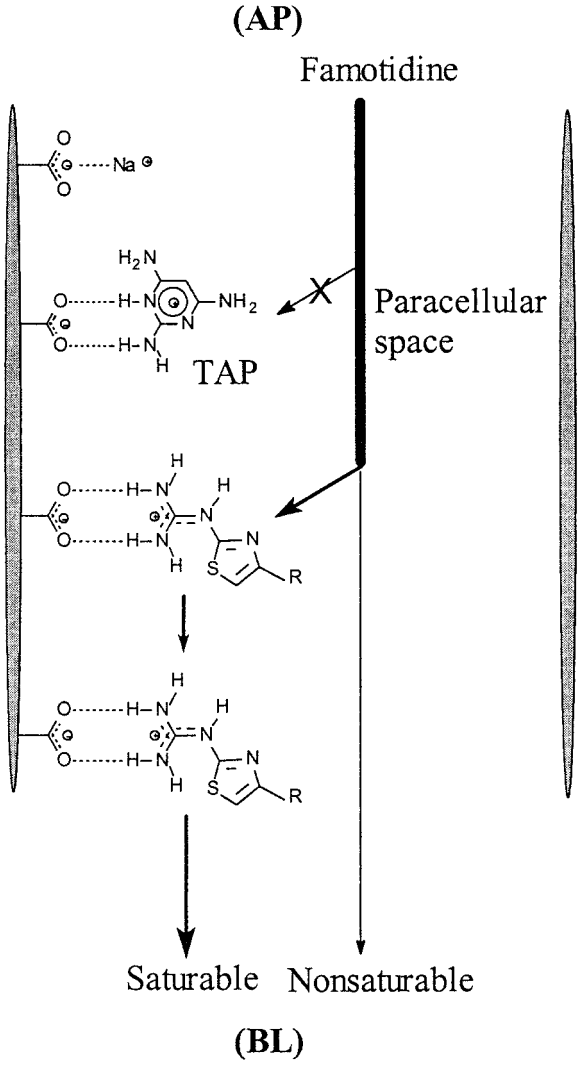

Figure 7—Model for the proposed transport mechanism of H_2-antagonists as exemplified by that for the transport of famotidine. Ionic interaction between Na^+ ions δ and the negatively charged group (as exemplified by carboxylate group here) in the paracellular space mediates cation-selective ion transport.[21] Likewise, the positively charged species of famotidine is transported by facilitated diffusion through interaction with the negatively charged group in the paracellular space (saturable transport). Famotidine can also traverse the paracellular space by simple diffusion (nonsaturable transport). This figure also shows how a cationic compound such as 2,4,6-triaminopyrimidine (TAP) can compete for the anionic site and inhibit the transport of famotidine (or ranitidine).

monolayers in the same way the transport of Na^+ ions is assisted by interactions with the anionic sites. Our results show that this process is not likely to be an active transport process; rather it can be characterized as facilitated diffusion. A population of ranitidine (and famotidine) molecules that do not interact with the anionic sites would traverse the paracellular space by a passive diffusion process (Figure 7). The overall transport of these compounds is thus a sum of the saturable facilitated diffusion process and nonsaturable passive diffusion process. The proposed mechanism is consistent with the observed transport kinetics for ranitidine and famotidine, inhibition of their own transport at high concentration, and inhibition of their transport by cationic compounds such as TAP. The possible involvement of the paracellular anionic sites in the drug transport has been also suggested in some recent reports[26–28] based on the higher paracellular permeability of cationic compounds compared to that of anionic compounds. However, this is the first report in which saturable transport of cationic hydrophilic compounds across epithelial cells has been described that can be explained by a (proposed) molecular mechanism involving their interactions with the anionic sites in the paracellular space. We want to emphasize that other mechanisms can be proposed to explain the observed saturable kinetics for the transport of ranitidine and famotidine across Caco-2 cell monoalyers; however, the mechanism proposed here serves as a good working model to help design further studies[29] that can provide greater insights into the mechanism of intestinal transport for hydrophilic cationic compounds such as ranitidine and famotidine.

The results presented here and the proposed mechanism for transport of ranitidine and famotidine raise an interesting possibility of potential drug interactions and food effects, because the oral absorption of cationic hydrophilic compounds is likely to be affected by coadministered drugs with similar structural characteristics and by hydrophilic cationic peptides generated from hydrolytic digestion of proteins in the diet.[30]

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