pH-Dependent Bidirectional Transport of Weakly Basic Drugs across Caco-2 Monolayers: Implications for Drug–Drug Interactions

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Purpose. The purpose of this study was to investigate the pH-dependent passive and active transport of weakly basic drugs across the human intestinal epithelium.

Methods. The bidirectional pH-dependent transport of weak bases was studied in Caco-2 cell monolayers in the physiologic pH range of the gastrointestinal tract.

Results. A net secretion of atenolol and metoprolol was observed when a pH gradient was applied. However, the bidirectional transport of both compounds was equal in the nongradient system. Hence, at lower apical than basolateral pH a change in passive transport caused by an imbalance in the concentration of the uncharged drug species resulted in a "false" asymmetry (efflux ratio). Furthermore, a mixture of pH-dependent passive and active efflux was found for the P-glycoprotein (P-gp, MDR1, ABCB1) substrates, talinolol and quinidine, but not for the neutral drug, digoxin. However, the clinically important digoxin–quinidine interaction depended on the presence of a pH gradient. Hence, the degree of interaction depends on the amount of quinidine available at the binding site of the P-gp.

Conclusions. Active efflux of weak bases can only be accounted for when the fraction of unionized drug species is equal in all compartments because the transport is biased by a pH-dependent passive component. However, this component may take part *in vivo* and contribute to drug–drug interactions involving P-gp.

KEY WORDS: P-glycoprotein; pH dependency; passive and active efflux; drug absorption; Caco-2; ABCB1.

INTRODUCTION

Most drug-like compounds that are generated by drug discovery programs become ionized in the physiologic pH range that is present in the gastrointestinal (GI) tract in a manner that depends on their pK_as (1,2). This is also the case for drugs on the market. The membrane permeability of the uncharged species of a drug molecule is much higher than that of the charged species, and local changes in the intestinal pH may have significant effects on the absorption of the drug molecule (3,4).

Surprisingly, there are few studies dealing with pH-dependent absorption of orally administered drugs *in vivo*. These studies mainly support the pH-partitioning theory, which states that only the uncharged species of a compound can permeate the membrane and that the permeability of a drug is therefore a function of the concentration of the uncharged species (1). Deviations from the pH-partitioning theory have been attributed to the presence of an acidic microclimate adjacent to the apical surface of the intestinal epithelium and to effects of the aqueous boundary layer (5). However, our recent *in vitro* studies using Caco-2 cell monolayers (6) indicated that transport of a charged species of a drug molecule across the intestinal epithelium can be significant, presumably via the paracellular pathway. It was found, in general, that the contribution of the charged species to the overall permeability is significant when the uncharged fraction of a drug molecule is less than 0.1 over the entire physiologic pH range (6). Our previous study had two limitations. First, we investigated only bases, having pK_a values around 6.5, whereas most drugs are weak bases with much higher pK_a values (7). Examination of an in-house database (AstraZeneca, Mölndal), comprising a total of 618 registered oral drugs (*Physicians Desk Reference*, 1999) reveals how prevalent weak bases are among drugs. Classification of these drugs into subgroups, such as various bases, acids, zwitterions, and neutral compounds, shows that approximately 40% of these drugs are amines and are thus weak bases. Second, we studied only passive diffusion and thus obtained no information on the pH dependency of active transport. Therefore, this study was designed to investigate the pH-dependent permeability and the active efflux of drugs that are weak bases.

For this purpose, we chose human intestinal epithelial Caco-2 cell monolayers as a transport model for the following reasons: 1) Caco-2 cell monolayers remain intact at apical pH values that cover the entire physiologic range of the intestinal tract (pH 5–8; Refs. 6,8,9); 2) the monolayers do not have rate-limiting subepithelial tissues, which makes them suitable for studies of bidirectional drug transport; and 3) they lack a protective mucus layer, which means that the pH at the cell surface rapidly equilibrates and becomes equal to the bulk pH of the applied buffer solution. Two types of drugs were studied: drugs that permeate the cell membrane solely by a passive mechanism, and drugs that are known to be substrates of the active efflux protein, P-gp. In addition, the pH dependence of clinically relevant drug–drug interactions involving P-gp are reported.

MATERIALS AND METHODS

Selection of Model Drugs

Several criteria were used to select drugs for inclusion in the study, the most important of which was that the drugs must be weakly basic. Drugs must also be chemically and metabolically stable in the GI tract. Drugs were classified as either poorly permeable or highly permeable using polar surface area and lipophilicity to predict their permeabilities (4,10). One poorly permeable drug that is predominantly passively transported across the cell membrane, atenolol, was selected, and one drug that is highly permeable, metoprolol, was chosen. We noted that atenolol showed a slightly faster transport in the upper GI tract *in vivo* (4), indicating a role of the paracellular route. However, adaptation of the calcula-

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tions by Palm *et al.* (6) revealed that the contribution of the paracellular pathway to the overall transport of atenolol was less than 20% in the Caco-2 cell model and the transcellular route therefore dominated. Thus, the calculations showed that atenolol is a suitable model drug for the purpose of this study.

Two drugs were selected that are actively effluxed from cells via P-gp: talinolol, which is poorly permeable, and quinidine, which is highly permeable. One additional drug, the neutral P-gp substrate digoxin, was included in the study. This allowed us to study the effects of the luminal pH on the efflux mediated by P-gp. The inclusion of digoxin in the study also made it possible to investigate the influence of the luminal pH on the fatal drug-drug interaction between digoxin and quinidine (11), and the recently observed interaction between digoxin and talinolol (12). Preliminary experiments on the concentration-dependent transport of these compounds confirmed that atenolol and metoprolol are transported passively and that talinolol, quinidine, and digoxin are actively secreted to the apical side of the monolayers (data not shown).

The polar surface area and the nonpolar surface area of the compounds were calculated using the program SaSA (version 0.8, Olsson, T.; Sherbukhin, V., Synthesis and Structure Administration, 1997-2001, AstraZeneca). Values for LogP (ACDLogP) and pK_a (ACDpK_a) were calculated using the ACDlabs databases version 4.56 (Advanced Chemistry Development Inc., Toronto, Canada; Table I). Experimentally determined LogP and pK_a values were obtained from the literature (Table I; Ref. 13).

Compounds and Radiolabeled Markers

[^{3}H]-Digoxin (629 GBq/mmol) and [^{14}C]-mannitol (1.9 GBq/mmol) were purchased from NEN™ Life Science Products Inc. (Boston, MA, USA). Quinidine [9-^{3}H] (740 GBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). [^{3}H]-Metoprolol (31.5 GBq/mmol), [^{3}H]-atenolol (229 GBq/mmol), talinolol, and [^{3}H]-talinolol (639 GBq/mmol) were synthesized at AstraZeneca (Mölndal, Sweden). OptiPhase 'Highsafe' 3 was purchased from Wallac (Loughborough, UK) and 2-morpholinoethanesulfonic acid monohydrate (Mes) was obtained from Fluka (Gothenburg, Sweden). All other chemicals were obtained from Sigma-Aldrich Co. (Stockholm, Sweden).

Cell Culture

Caco-2 cells, obtained from ATCC (Rockville, MD, USA) at passage 18, were maintained as described previously (14) using heat-inactivated fetal calf serum. We used Caco-2 cells in the same conditions as those used for screening at AstraZeneca (Mölndal, Sweden): at passage 27–41 and routinely seeded at a density of 3.5×10^6 cells per 175 cm^2-flasks. The cells were grown to 90% confluence and harvested by regular trypsinization using a trypsin (0.05%)–EDTA (0.02%) solution. The medium was changed every second day. For transport experiments, 2.56×10^5 cells were seeded onto each 12-mm polycarbonate cell culture insert – 12 well plates with an area of 1.13 cm^2 and a pore size of 0.4 μm (Transwell®, Cat. No. 3401, Corning Costar® Corporation, Cambridge, MA, USA). The cells on the plates were maintained, using culture medium containing antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) in an atmosphere of 5% CO_2. The relative humidity was 90%. All tissue culture media were obtained from GIBCO, Life Technologies (Paisley, Scotland).

Transport Studies

Transport studies were performed as previously described (6) with slight modifications. Briefly, bidirectional transport rates (apical-to-basolateral: a-b; basolateral-to-apical: b-a) of the test compounds were measured using Caco-2 monolayer cultures grown in the Transwell® system for 23 ± 1 days. Each experiment was performed at least in triplicates. The incubation medium was Hank's balanced salt solution (HBSS) buffered either with 10 mM Mes (pH 5.0–6.5), or with 25 mM HEPES (pH 7.0–8.0). All solutions were prewarmed to 37°C. The culture medium was removed from both sides of the monolayers and they were washed with the desired HBSS-buffer. The cell monolayers were then preincubated for 30 min without the test compound, and then incubated for 25 min at 37°C with the test compound (at concentrations from 2 nM to 50 μM, depending on the compound). The volumes of the appropriate incubation medium used were 0.5 or 1.5 mL, and the pH values used in the apical compartment were 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, 7.7, and 8.0, whereas those used in the basolateral compartment were 6.5 and 7.4. Samples were withdrawn to determine the initial and

Table I. Physicochemical Properties and Oral Fraction Absorbed (fa) of the Drugs

Compound	ACDpK_a[a]	pK_a[b] exp.	ACDLog P[a]	Log $P_{o/w}$[c] meas.	PSA [Å^2][d]	NPSA [Å^2]	Ratio NPSA/PSA	Molecular weight	fa[e] [%]
Atenolol	9.17	9.6	0.10 ± 0.25	0.16	92.5	255.4	2.8	266.34	50
Metoprolol	9.18	9.7	1.79 ± 0.23	1.88	55.0	307.3	5.6	267.37	80–100
Talinolol	9.17	n.d.	3.20 ± 0.30	—	93.4	383.6	4.1	363.50	40–70
Quinidine	9.13	4.2; 7.9	3.36 ± 0.43	3.44	43.1	334.3	7.8	324.43	100
Digoxin	—	n.a.	1.14 ± 0.84	1.26	215.2	676.4	3.1	780.96	40–80

The molecular surface is described by the polar surface area (PSA) and the nonpolar surface area (NPSA).
n.d. = not determined; n.a. = not applicable.
[a] Values for logP and pK_a were calculated using the ACD database.
[b] Experimentally determined pK_a values (from Ref. 13).
[c] Octanol/water partition coefficients (from Ref. 13).
[d] Ångström (Å), 1 Å = 10^{-10} m = 0.1 nm.
[e] Values from Martindale, *The Complete Drug Reference,* 32nd ed.

final donor concentration of the compound and for calculation of the mass balance. The receiver medium was totally replaced after 5, 15, and 25 min. For a-b transport, 1.5 mL of medium from the basolateral side was removed, whereas for b-a transport, 400 μL of medium from the apical side was removed. (The initial volume on the apical side was 500 μL).

The amount of drug transported was determined from the radioactivity content in the samples using a scintillation counter (Wallac, Turku, Finland). Monolayer permeability for the paracellular marker mannitol was determined simultaneously. The monolayers were placed onto a calibrated plate shaker (BMG LabTechnologies GmbH, Offenburg, Germany) set at a high stirring rate (450 rpm) throughout the experiment to minimize the influence of the aqueous boundary layer. The initial values of transepithelial electrical resistance (TER) were measured with an epithelial volt-ohmmeter, equipped with an SX-2 electrode (World Precision Instruments Inc., Sarasota, FL, USA).

Low concentrations (2 nM–50 μM) of all drugs were used to avoid toxicity and solubility problems. The absence of any toxic effect of the drugs was confirmed by measuring the monolayer integrity using mannitol and TER. The concentration that was required in the drug–drug interaction studies to mimic the luminal drug concentration *in vivo* as closely as possible was determined from the following reasoning. Digoxin is given orally at a low dose of 0.1 mg per day. This dose gives a concentration of 500 nM when dissolved in 250 mL of water. Similarly, quinidine is given orally at a low dose of 1000 mg daily, giving a concentration of 12.33 mM when dissolved in 250 mL of water. Finally, talinolol is given at a low dose of 230 mg per day, giving a concentration of 2.5 mM when dissolved in 250 mL of water. Thus, the simultaneous administration of digoxin and quinidine results in a molar dose ratio of 1:25,000. The corresponding molar dose ratio for digoxin and talinolol is 1:5,000. Consequently, the pH-dependent drug-drug interactions of the compounds were studied at these ratios, after taking into account the toxicity limit for the monolayers. Accordingly, we used a concentration of digoxin of 2 nM, a concentration of quinidine of 50 μM, and a concentration of talinolol of 10 μM reflecting the expected relative concentrations under physiologic conditions, based on common doses used in clinical practice. All compounds were used at concentrations above their maximal plasma concentration (C_{max}; Refs. 15–17).

Data Treatment

All experiments were performed under "sink" conditions, and this allowed the apparent permeability coefficients (P_{app}) to be calculated as described previously (18,19). The flux of all test compounds was linear with time under all conditions ($r^2 \geq 0.98$). The ratio of the transport in the b-a to that in the a-b direction was calculated in order to obtain information regarding any asymmetry in the transport of the drug. Thus, (apical) drug efflux ratios were calculated using the following equation:

$$\text{Efflux Ratio}_{\text{apical pH/basolateral pH}} = P_{app(b\text{-}a)} / P_{app(a\text{-}b)} \quad (1)$$

Values are expressed as the means of at least three experiments ± standard deviation (SD). The statistical difference between the permeabilities of the drugs at different pH gradients was calculated using an unpaired *t* test with a two-tailed distribution. A p value less than 0.01 was considered to be statistically significant.

RESULTS

Integrity of Caco-2 Monolayers

P_{app} for the membrane integrity marker mannitol was the same ($p > 0.05$) at the different pH gradients and for both transport directions studied ($P_{app} = 0.36 \pm 0.11 \times 10^{-6}$ cm s^{-1}; $n = 517$). The TER of the Caco-2 cell monolayers increased during the growth of the cells, from 44 ± 28 Ω cm^2 on the second day after seeding to 280 ± 40 Ω cm^2 on the tenth day, and remained constant thereafter ($n = 62$). The TER values remained constant under all investigated pH-conditions and were not influenced by the transport direction ($n = 48$-56 for each compound). The bidirectional transport of the paracellular marker mannitol and the TER in the present study indicated that the epithelia did not leak and that the transport of mannitol was consistent with purely passive diffusion, independent of the pH. This agrees with earlier reports (6,8,9,14,20,21). Thus, the pH-dependent change in drug permeability observed here was due solely to a difference in the unionized fraction of the bases and not because of the effects of pH on the monolayer integrity.

pH-Dependent Permeability of Weak Bases That Are Passively Transported

The pH-dependent permeabilities of the two passively transported weak bases, atenolol and metoprolol, were studied first. Transport of the poorly permeable drug, atenolol, decreased by a factor of eight (from $2.31 \pm 0.09 \times 10^{-6}$ cm s^{-1} to $0.30 \pm 0.08 \times 10^{-6}$ cm s^{-1}) in the a-b direction, and transport in the b-a direction was not affected when the apical pH was reduced from pH 8.0 to 5.0 (Fig. 1A). Transport of the highly permeable compound, metoprolol, in a-b direction decreased by a larger factor (50 times), from $186 \pm 6 \times 10^{-6}$ cm s^{-1} to $3.9 \pm 0.2 \times 10^{-6}$ cm s^{-1} (Fig. 1B). Transport of metoprolol in the b-a direction was significantly affected by the pH on the apical side, (from $62 \pm 3 \times 10^{-6}$ cm s^{-1} to $142 \pm 9 \times 10^{-6}$ cm s^{-1}; Fig. 1B).

Experiments using Caco-2 are often conducted using a pH gradient in which the pH on the apical side is 6.5 (8). We therefore determined the rates of pH-dependent passive efflux under this pH gradient and compared them to the rates obtained when the pH on both sides is equal (pH 7.4/7.4 and pH 6.5/6.5). At equal pH-values the bidirectional permeabilities for atenolol and metoprolol were not significantly different (Table II; Fig. 2A and B). The permeabilities were lower at pH 6.5 than those at pH 7.4 (Fig. 2D and E). In contrast, the a-b transport rates were lower and the b-a transport rates were higher, when a pH gradient (apical: 6.5; basolateral: 7.4) was present, revealing that the observed efflux for atenolol and metoprolol (efflux ratio 1.7 ± 0.2 and 4.5 ± 0.4, respectively) are indeed passive effluxes (Fig. 2C and F). Thus, passive (pseudo-) efflux can occur across Caco-2 cell monolayers under certain experimental conditions.

pH-Dependent Permeability of Weak Bases That Are Actively Effluxed

We then determined the rate of a-b transport of the poorly and highly permeable P-gp substrates, talinolol and

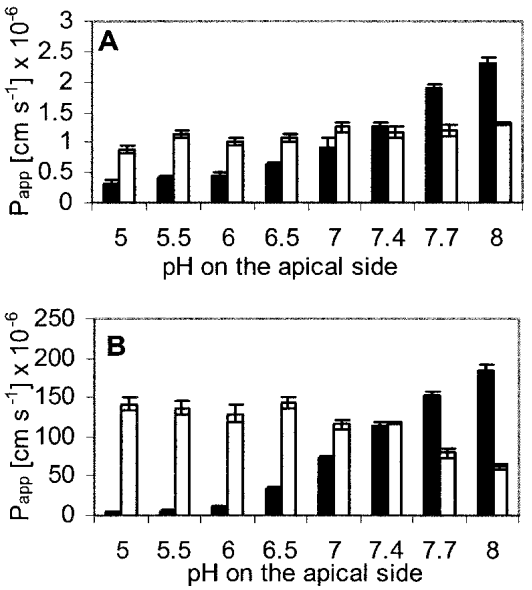

Fig. 1. Bidirectional pH-dependent transport across Caco-2 monolayers of (A) the slowly transported weak base, atenolol, and (B) the rapidly transported weak base, metoprolol. The apparent permeability coefficient (P_{app}) in the apical-to-basolateral direction (black bars) increased with apical pH. The basolateral pH was always 7.4. The P_{app} in the basolateral-to-apical direction (white bars) decreased for metoprolol and remained unchanged for atenolol as the apical pH increased. Each bar indicates mean ± SD, ($n \geq 3$).

quinidine. The permeability of talinolol decreased by a factor of 2.6 (from 7.73 ± 0.32 × 10^{-6} cm s^{-1} to 2.96 ± 0.08 × 10^{-6} cm s^{-1}) when the apical pH was reduced from pH 8.0 to 5.0 (Fig. 3A), whereas that of quinidine decreased by a factor of 68 (from 63.02 ± 1.75 × 10^{-6} cm s^{-1} to 0.93 ± 0.06 × 10^{-6} cm s^{-1}; Fig. 3B). In contrast, the b-a transport was significantly increased for both compounds under the same conditions. The increase that was more pronounced for the highly permeable drug quinidine (from 99.73 ± 22.48 × 10^{-6} cm s^{-1} to 165.67 ± 14.09 × 10^{-6} cm s^{-1}), than it was for the poorly permeable drug talinolol (from 20.86 ± 1.87 × 10^{-6} cm s^{-1} to 34.63 ± 1.94 × 10^{-6} cm s^{-1}; Fig. 3A and B). Hence, the efflux ratio for talinolol (at a concentration of 58 nM; 37 kBq [^{3}H]-talinolol per mL) ranged from 12 to 3, whereas the efflux ratio for quinidine (at a concentration of 50 nM; 37 kBq [^{3}H]-quinidine per mL) covered a wider range from 178 to 1.6. In summary, the Caco-2 cell monolayers were more permeable for talinolol and for quinidine in the b-a direction than in the a-b direction, under all conditions (Fig. 3).

The rates of pH-dependent passive efflux of these two drugs at an apical pH of 6.5 were compared with those obtained when the pH values on both sides of the monolayers were equal (Fig. 4). The permeabilities for talinolol and quinidine were significantly different in the different transport directions at equal pH-values (Figs. 4A and B), resulting in efflux ratios of 6.0 and 4.0, respectively (Table II). These ratios reflect the active P-gp-mediated efflux. The a-b transport rates were even lower and the b-a transport rates were even higher, when cells were exposed to the pH gradient (apical: 6.5; basolateral: 7.4), showing that the transport contains a significant component of passive (pH dependent) efflux. The resulting efflux ratios, which are comprised of both passive and active efflux components, were 8 and 20, for talinolol and for quinidine, respectively. Thus, passive efflux may contribute significantly to drug efflux at certain values of the local pH-gradient.

Lack of pH-Dependent Efflux of Digoxin: A Neutral P-gp Substrate

The efflux ratio of the neutral P-gp substrate, digoxin, did not depend on the pH on the apical side of the membrane (Fig. 5A), while those for quinidine and talinolol did. The P_{app} values revealed that a (b-a)-directed active transport system is involved in the transport of digoxin (at a concentration of 59 nM; 37 kBq [^{3}H]-digoxin per mL). Thus, the rate of b-a transport was higher (37 ± 3 × 10^{-6} cm s^{-1}) than that of a-b transport (4.2 ± 0.5 × 10^{-6} cm s^{-1}; Fig. 5A). The average efflux ratio for all pH gradients were 8.9 ± 0.8, indicating that the activity of P-gp is constant in the entire pH interval studied.

pH Dependence of Clinically Relevant Drug–Drug Interactions

Drug–drug interactions between digoxin and other P-gp substrates, such as quinidine and talinolol are interesting because digoxin has a narrow therapeutic window. Transport of digoxin alone was independent of the buffer pH (Fig. 5A), whereas the bidirectional transport of digoxin in the presence of quinidine depended on the pH (Fig. 5B). However, in the presence of talinolol was only the a-b transport of digoxin affected by pH (Fig. 5C). The effects of both talinolol and quinidine on digoxin transport were greater at higher apical pH values. This effect might be caused by a larger uncharged fraction of the basic drug at high pH values. This in turn, would result in increased membrane partition and permeability and, thus, a higher concentration of the basic drug would be available at the P-gp binding site, reducing the digoxin efflux.

DISCUSSION

This is the first report that investigates the pH dependence of both passive and active efflux of orally administered basic drugs across the intestinal epithelium. The pH dependence of drug–drug interactions involving P-gp is here also reported for the first time. Our results have important implications for the interpretation of *in vitro* studies of passive and active drug transport and efflux, and they may have important implications for the interpretation of *in vivo* studies.

The efflux ratios for atenolol and metoprolol were considerably greater than 1.0 when the commonly used pH gradient of 6.5 (apical) and 7.4 (basolateral) was used, even though these drugs are passively transported across Caco-2 cells (18). The efflux ratio was equal to 1.0, however, in the absence of a pH gradient, which shows that these drugs are not actively transported under the applied experimental conditions. These results support the pH-partitioning theory (1). This theory states that the fraction ionized of a basic drug decreases from pH 6.5 to pH 7.4 and that the concentration of uncharged drug species (which drives the passive transmembrane transport of the drug) is therefore lower at pH 6.5. Thus, the theory predicts that the permeability of a basic drug in the a-b direction is lower at an apical pH of 6.5 than it is at an apical pH of 7.4, and our results agree with this prediction.

Table II. Permeability of Caco-2 Cell Monolayers and Efflux Ratios (ER) for the Investigated Compounds

Compound	$P_{app\ (a\text{-}b)}$ [cm s^{-1}] × 10^{-6}	$P_{app\ (b\text{-}a)}$ [cm s^{-1}] × 10^{-6}	Permeability high/low	$ER_{7.4/7.4}$	Efflux yes/no
Atenolol	1.26 ± 0.08	1.17 ± 0.10	Low	0.93	No
Metoprolol	116.2 ± 2.2	107.9 ± 7.3	High	0.93	No
Talinolol	4.13 ± 0.40	24.8 ± 1.9	Low	6.0[a]	Yes
Quinidine	30.4 ± 1.6	122.3 ± 8.7	High	4.0[a]	Yes
Digoxin	4.8 ± 0.2	40.4 ± 2.6	High	8.4[a]	Yes

Note: The apparent permeability coefficients in the apical-to-basolateral ($P_{app\ (a\text{-}b)}$) and basolateral-to-apical ($P_{app\ (b\text{-}a)}$) directions represent the values obtained using the non-gradient system, in which a pH value of 7.4 is used both on the apical and the basolateral sides of the monolayers. Values are mean ± SD, (n ≥ 3).
[a] Concentration and cell age dependent.

The pH-dependent passive permeability of weak bases has also been observed in the perfused rat intestine *in situ* (4). Consistent with the pH-partitioning hypothesis, the ionized fraction of basic molecules is lower in the rat ileum (pH 7.3) than in the rat jejunum (pH 6.5), and that bases are absorbed more rapidly in the lower parts of the GI tract (4). A lower pH (pH 6.6) adjacent to the epithelial cells has also been observed in the upper small intestine in humans (3). This acid microclimate will determine the molecular charge of the drug before it is absorbed across the intestinal wall. Since the jejunum is the major absorption site for most drugs in humans, several researchers have recommended that studies of drug permeability across Caco-2 monolayers should be performed using a pH gradient in which the pH is 6.0–6.5 on the apical side and 7.4 on the basolateral side. This recommendation is supported by results indicating that permeability data obtained with this pH gradient give slightly better correlations to the fraction of the oral dose absorbed in man (8,22) than values obtained without a pH gradient. This has also been reported for jejunal permeability measured in perfusion studies in healthy human subjects (performed at pH 6.5; Ref. 23). Although a pH-gradient system might be advantageous in screening of passive Caco-2 cell permeability, it will not be conclusive according to our results as regards the prediction of the active drug efflux component (e.g., P-gp).

The rates of efflux of talinolol and quinidine, two P-gp substrates (12,24), were significant under all the experimental conditions used. This contrasts with the results obtained for atenolol and metoprolol. In agreement with findings for atenolol and metoprolol, comparable pH-dependent changes in

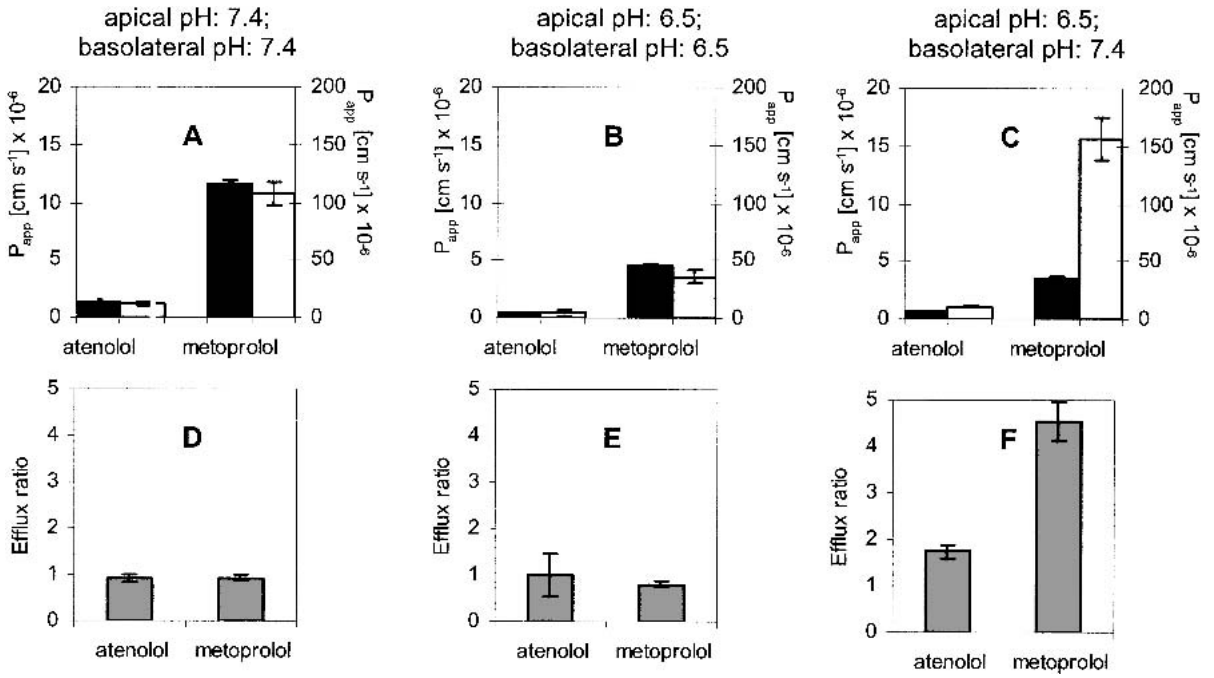

Fig. 2. The apparent permeability coefficients (P_{app}) of the slowly transported weak base, atenolol (first y-axis) and the rapidly transported weak base, metoprolol (second y-axis) across Caco-2 monolayers were equal in both transport directions using a buffer pH of (A) 7.4 or (B) 6.5 on both sides. (C) In the presence of a pH gradient (apical pH 6.5 and basolateral pH 7.4), the basolateral-to-apical transport exceeded the transport in the apical-to-basolateral direction. The corresponding efflux ratios at a buffer pH of (D) 7.4 and (E) 6.5 on both sides of the monolayers and (F) at the pH gradient (apical/basolateral 6.5/7.4) indicate that a net efflux (a false active efflux) in the basolateral-to-apical direction was obtained in the presence of a pH gradient (apical pH 6.5 and basolateral pH 7.4). Values are mean ± SD, n ≥ 4.

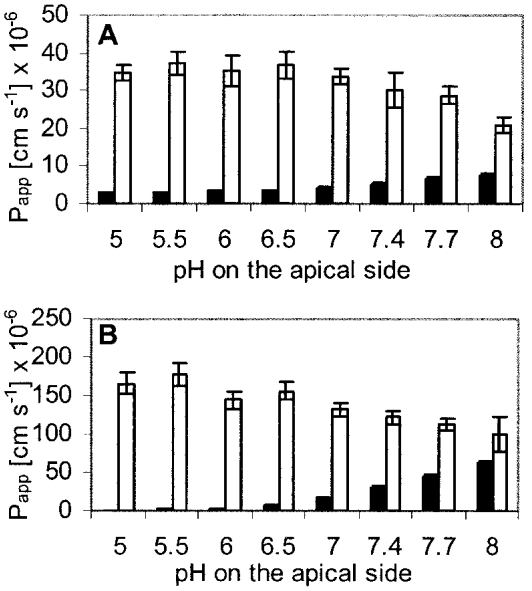

Fig. 3. Bidirectional pH-dependent transport across Caco-2 monolayers of two P-glycoprotein substrates (A) the slowly transported weak base, talinolol, and (B) the rapidly transported weak base, quinidine. The apparent permeability coefficient (P_{app}) in the apical-to-basolateral direction (black bars) increased and the P_{app} in the basolateral-to-apical (white bars) decreased as the apical pH increased. The basolateral pH was always 7.4. Both P-glycoprotein substrates show a net efflux over the whole pH range, consistent with an unsaturated apical efflux transported. Each bar indicates mean ± SD, n ≥ 4.

passive permeability occurred also for the basic drugs talinolol and quinidine. These changes meant that the relative contribution of the active and passive transport components was varying under the different pH conditions. It is believed that permeation of a drug through, or at least into, the lipid membrane is required for it to gain access to the binding sites of P-gp (25,26). This means that a high apical pH, which increases the fraction of uncharged drug and hence membrane partitioning, will increase the probability that a weakly basic drug will interact with P-gp binding sites. However, our results demonstrate that the b-a efflux for both talinolol and quinidine were lower at higher pH values. At the same time, the a-b permeability increased, probably as a result of an increased passive diffusion of the uncharged species in the a-b direction. Understanding and delineating this complex relationship will require measurements of intracellular pH, and it will require measurement of the intracellular and (possibly) the intramembrane concentrations of the drugs. Moreover, the involvement of other active transport processes such as organic cation transporters (e.g. OCT, OATP) might have an impact on the permeability of the basic compounds used in this study (27). Double-transfected cells expressing two or more active transporters (28,29) as well as more specific substrates will help to address these issues in the future.

The here presented data point out that the pH-dependent passive permeability must be considered in studies of the active efflux of weak bases in transport models such as Caco-2. The passive permeability influences the rate of drug transport in both directions across the cell monolayer and hence the observed efflux ratio. As a result, *in vitro* data obtained with Caco-2 monolayers may over-emphasize the

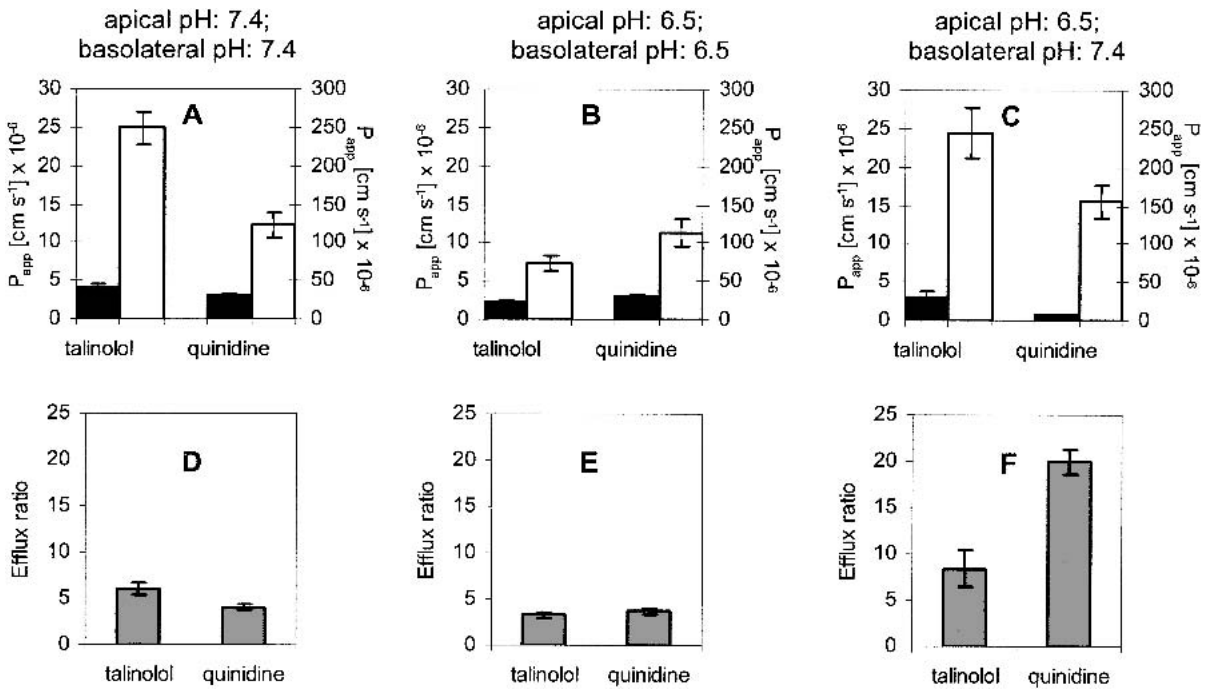

Fig. 4. Apparent permeability coefficients (P_{app}) across Caco-2 monolayers in basolateral-to-apical direction of the slowly transported weak base and the P-glycoprotein substrate, talinolol (first y-axis) and the rapidly transported weak base and the P-glycoprotein substrate, quinidine (second y-axis), always exceeded the corresponding P_{app} in the apical-to-basolateral direction using a buffer pH of (A) 7.4 or (B) 6.5 on both sides, and (C) in the presence of a pH gradient (apical pH 6.5 and basolateral pH 7.4). The corresponding efflux ratios at a buffer pH of (D) 7.4 and (E) 6.5 on both sides of the monolayers and (F) using the pH gradient (apical/basolateral: 6.5/7.4) are also shown. Values are mean ± SD, n ≥ 4.

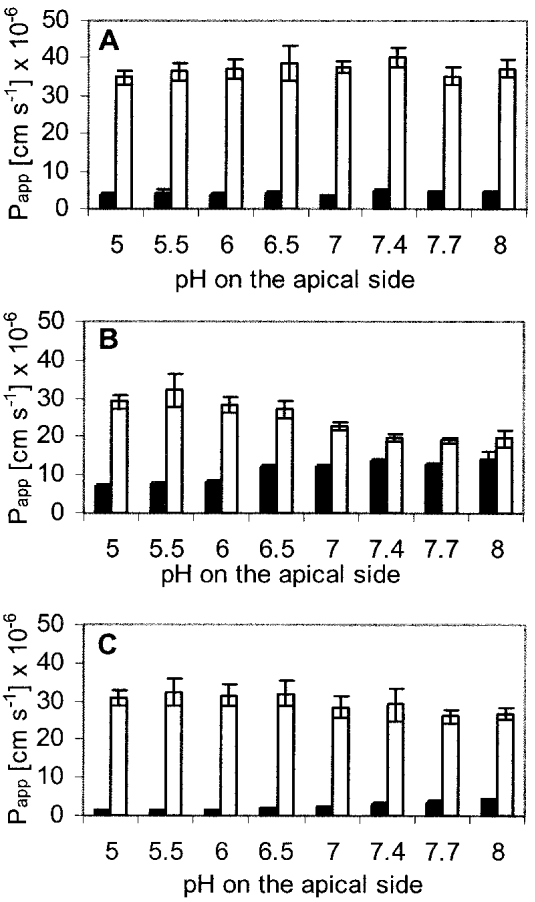

Fig. 5. A, Apparent permeability coefficients (P_{app}) of the neutral P-glycoprotein substrate, digoxin, in the apical-to-basolateral direction (black bars) and in the basolateral-to-apical direction (white bars) remained unchanged as the apical pH increased. The basolateral pH was always 7.4. Constant efflux ratios (8.9 ± 0.8; n = 90) were obtained, reflecting the efflux of the neutral drug digoxin by a transporter, having an activity that is independent of a pH gradient. pH-Dependent permeability values for bidirectional transport of the neutral P-glycoprotein substrate, digoxin (2 nM), in the presence of (B) 50 μM quinidine or (C) 10 μM talinolol. Donor concentrations are estimated from the concentration ratio after oral administration (see Methods section). Values are mean ± SD, n ≥ 3.

contribution of efflux systems such as P-gp. The transporter will not be saturated at sufficiently low initial donor concentrations, and any involvement of an efflux system in the transport of a basic drug will be evident. Our *in vitro* findings can be extrapolated to the *in vivo* situation. In this case, the regional absorption of basic P-gp substrates will vary not only with variations in the regional expression of functional P-gp along the GI tract, but also with variations in the regional pH adjacent to the apical cell membrane of the intestinal villus enterocyte.

Digoxin is a neutral substrate of P-gp (30), and we used this drug in an attempt to eliminate some of the complexity associated with the study of the pH-dependent active transport. Interestingly, the efflux ratio of digoxin was more or less constant at all of the investigated pH gradients, indicating that its active efflux was independent of the apical pH. Digoxin is a neutral drug, and so a given concentration of digoxin will drive its permeation across the cell membrane independently of the extracellular pH. This means that the same amount of drug will be presented to the binding sites of P-gp under all the conditions that we investigated. The digoxin studies reveal that the activity of P-gp does neither depend on the extracellular proton concentration nor a proton gradient. This suggests that the P-gp activity is not directly regulated by pH changes. This hypothesis is supported by studies showing that a change in the intracellular pH does not affect the activity of P-gp (31,32). However, it has recently been suggested that the intracellular phosphate release upon P-gp-ATPase activation is tightly coupled to the rate of extracellular proton extrusion (33), although this increase in extracellular proton concentration only leads to a minor change in the pH of the extracellular medium. Further studies are needed before firm conclusions regarding the pH dependence of P-gp activity can be drawn.

The interaction between digoxin and P-gp was independent of the extracellular pH, and this made it possible to investigate pH-dependent drug–drug interactions using weakly basic P-gp substrates. Digoxin is an excellent choice for such studies, since it displays several clinical drug–drug interactions at the P-gp level, including those with quinidine and talinolol (11,12), and also since the expression level of a polymorphic form of P-gp has been linked to altered pharmacokinetics of digoxin in man (34,35). Thus, digoxin coadministration with P-gp inhibitors such as quinidine, atorvastatin and verapamil results in increased plasma levels of digoxin, even to clinically significant levels in the case of quinidine and verapamil (11,15,36). Our results revealed a strong pH-dependency for the digoxin-quinidine interaction and a significant, but less prominent interaction between digoxin and talinolol. This means that the pH-dependent transport of quinidine and talinolol across Caco-2 cell monolayers reflects pH-dependent base-digoxin interactions with P-gp. The degree of digoxin interaction with inhibitors of P-gp (such as quinidine, talinolol, verapamil) may also be dosage form dependent (37), and the interaction risk for bases may increase, along with the change of the local pH of the intestinal microclimate. This has implications for dosage form development, e.g., for extended release forms, when a similar absorption is required throughout the GI tract. Although the *in vivo* importance of our findings remains to be shown, they provide "proof-of-concept" and have clear implications for the choice of experimental conditions in *in vitro* studies of drug-drug interactions in cell culture systems.

CONCLUSION

The results of this study indicate that passive bidirectional transport of weak bases across cell culture models such as Caco-2 cell monolayers is influenced by the pH conditions in a way that can lead to misinterpretation of the role of active drug transport. Consequently, drug efflux ratios obtained from a pH-gradient system can falsely indicate active apical efflux for weakly basic drugs in these *in vitro* systems. The pH adjacent to the epithelium varies along the intestinal tract, and thus pH-gradient effects similar to those seen *in vitro* (leading to "false" efflux ratios) may influence drug absorption *in vivo*. The function of the P-gp seems not to depend on pH, but the efficiency of the transporter depends on the amount of compound available, i.e., the fraction unionized on the luminal side of the enterocyte. Our studies show that this pH-dependence impacts clinically relevant (P-gp-dependent) drug–drug interactions and it should therefore be considered in the design of *in vitro* models of drug-drug interactions.

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