The aim of this study was to investigate pH-dependent passive and active transport of acidic drugs across Caco-2 cells. Therefore, the bidirectional pH-dependent transport of two acidic drugs, indomethacin and salicylic acid, across Caco-2 cells was studied in the physiological pH range of the gastrointestinal tract. The transport of both drugs decreased with increased pH, as expected from the pH-partition hypothesis. Net absorption occurred when the basolateral pH exceeded the apical pH. Concentration dependence and transporter inhibition studies indicated passive transport for indomethacin and a mixture of pH-dependent passive and active influx for salicylic acid. Unexpectedly, active and passive drug transport results were indistinguishable in temperature dependency studies. The transport of salicylic acid (apical pH 5.0; basolateral pH 7.4) was partly blocked by inhibitors of the proton-dependent transporters MCT1 (SLC16A1) and OATP-B (SLC21A9; SLCO2B1). This study shows that the asymmetry in bidirectional transport of acidic drugs is affected by both passive and active components in the presence of pH gradients across Caco-2 cells. Thus, combined studies of concentration-dependency and transport-inhibition are preferred when acidic drug transport is studied in a pH gradient. The findings of this in vitro study can be extrapolated to in vivo situations involving an acidic microclimate.

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Keywords: pH Dependency; Passive and active influx; Drug absorption; Caco-2; SLCO2B1; SLC21A9; OATP-B

1. Introduction

The luminal pH along the gastrointestinal (GI) tract varies between 5 and 8, while the intracellular pH and the pH of the blood are in the range of 7.2–7.4. Thus, along the small intestine, an inwardly directed pH gradient favors the uptake (influx) of acidic drugs into the blood. Acetylsalicylic acid, which is rapidly degraded to salicylic acid in the GI tract, and indomethacin are acidic drugs that diffuse rapidly across the epithelium and are therefore quickly and completely absorbed from the small intestine after oral administration. These drugs were used as model acidic drugs for this study.

The absorption profile of acidic drugs in the GI tract can in general be explained by the pH-partition hypothesis (Shore et al., 1957), which state that a lipid membrane allows the passage of uncharged but not charged drug species. However, there may be differences in the specific responses of passive and active transport mechanisms to the differences in pH. Several modifications of the pH-partition theory have consequently been proposed (Daniel et al., 1985; McEwan and Lucas, 1990). Additional factors potentially affecting absorption include: firstly, the influence of the acidic microclimate at the surface of the epithelium (Daniel et al., 1985; McEwan and Lucas, 1990); secondly, the presence of proton-mediated transport processes (Takagi et al., 1998; Tsuji et al., 1990); finally, the driving force of protein binding in the plasma (Colmenarejo et al., 2003; Yamashita et al., 2000). This study investigated the impact of the acidic microclimate...
and different pH gradients on the active and passive transport of acidic drugs across Caco-2 cell monolayers. In a forthcoming paper, we will report our investigations on the influence of plasma protein binding in the same system.

The acidic microclimate at the intestinal mucosal surface would increase the amount of uncharged weakly acidic drug available for passive diffusion (Daniel et al., 1985; McEwan and Lucas, 1990). This has been referred to as the pH-shift (Winne, 1977). However, weakly acidic drugs are not absorbed by passive diffusion alone. Although this is probably the main mechanism for most of these drugs, we now know that there are also several carrier-mediated transport mechanisms available in the mucosal and serosal membranes that are more or less specific for acidic compounds (Lee, 2000; Tsuji and Tamai, 1996).

Studies by Takanaga, Tsuji and Tamai suggested the involvement of a carrier-mediated transport system for monocarboxylic acids such as salicylic acid (Takanaga et al., 1994; Tsuji et al., 1990; Tsuji and Tamai, 1996). Brush border membrane vesicles, prepared from the small intestine of male rabbits (Tsuji et al., 1990), and cultured Caco-2 cell monolayers (Takanaga et al., 1994; Tsuji et al., 1994) were used to identify the mechanism behind the extensive salicylic acid absorption. It was shown that a Na⁺-gradient independent, but H⁺-gradient dependent cotransport system is involved in the transport of monocarboxylates. However, the relative contributions of this active influx to the overall transport remain unknown and the responsible transporters have been only partly identified.

Several Na⁺-independent H⁺-dependent cotransporters localized in the apical membrane of the GI tract are currently recognized. The most relevant of these are: the monocarboxylic acid transporter (MCT)-1 (SLC16A1), the H⁺-coupled oligopeptide transporter (PEPT)-1 (SLC15A1) and the organic anion transporting polypeptide (OATP)-B (SLC21A6; SLC22B1) (Herrera-Ruiz et al., 2001; Kobayashi et al., 2003; Stein et al., 2000). The proton-dependent MCTs (Halestrap and Price, 1999) are essential for the rapid transport of monocarboxylates such as lactate and pyruvate across the plasma membrane. Two isoforms of the MCT (MCT1 and MCT2) have been found in hamster caecum and human and pig colon. In the rat stomach, however, only MCT1 was found (Halestrap and Price, 1999). PEPT1, which is abundantly expressed in the human small intestine (Ganapathy et al., 1995), participates in the absorption of β-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors, anticancer drugs such as bestatin, and renin inhibitors (Tsuji and Tamai, 1996). The OATP group is a growing gene superfamily; these polypeptides mediate transport of a wide range of amphathptic organic solutes, such as bile acids, steroid conjugates, thyroid hormones and anionic oligopeptides, as well as drugs like digoxin and pravastatin (Hagenbuch and Meier, 2003). It has been shown recently that OATP-B is expressed in the apical membrane of human intestinal epithelial cells (Kobayashi et al., 2003). It thus appears to also play a role in the pH-dependent transport of anionic drugs in the human intestine (Kobayashi et al., 2003; Nozawa et al., 2004).

In summary, there are several underlying mechanisms operating in parallel which affect the absorption of acidic compounds, making the analysis of the transport of acidic drugs complex. Because a ‘false’ efflux ratio (passive efflux ratio) has been observed as a consequence of the pH dependency of weak bases in a pH-gradient system like the GI tract, one would also expect a ‘false’ passive influx ratio to occur for an acidic drug (Neuhoff et al., 2003). Since several proton-dependent mechanisms are involved in the absorption of acidic compounds, the observed influx ratios in permeability screening in in vitro cell culture models (e.g. Caco-2) may reflect not only passive but also active influx. However, to our knowledge, the relative impacts of passive and active, proton/pH-dependent influx mechanisms for acidic drugs have not been investigated previously. This is perhaps surprising, since both passive and active influx mechanisms are functional in vivo.

Our aim was therefore to investigate the relative contribution of these mechanisms to the influx of two acidic compounds, indomethacin and salicylic acid, under various pH conditions. In order to evaluate the relative significance of non-saturable and saturable transport, indomethacin was used as a representative of a predominantly passively transported acidic drug and salicylic acid was used as an acidic drug that is both passively and actively transported across the intestinal epithelium.

2. Material and methods

2.1. Selection of model drugs

The model acidic drugs, indomethacin and salicylic acid, were selected since they are sufficiently soluble, chemically and metabolically stable and ionizable within the physiological pH range of the GI tract. Although indomethacin is known to be an inhibitor of several transporters (Draper et al., 1997) [human organic cation transporter (hOCT)-1 (SLC22A1), and hOCT2 (SLC22A2) (Khamdang et al., 2002)], and a substrate of organic anion transporters (OAT)-1 (SLC22A6) and OAT3 (SLC22A8) (Khamdang et al., 2002), our preliminary experiments showed that indomethacin itself is not actively transported across Caco-2 cells (see Section 3). Salicylic acid, in addition to diffusing rapidly and passively across the cell membrane, is also transported via MCT1 (Takanaga et al., 1994) and is a substrate for several OATs (Khamdang et al., 2002). However, despite its low molecular weight, only 1% salicylic acid was reported to be transported paracellularly across the tight Caco-2 cell monolayer (Pade and Stavchansky, 1997), making salicylic acid a useful compound for the purpose of our study.
2.3. Compounds and radiolabeled markers

Table 1. Physicochemical properties and oral fraction absorbed (fa) of the drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Log P&lt;sub&gt;oct/wat&lt;/sub&gt;</th>
<th>PSA (Å&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>NPSA (Å&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Ratio NPSA/PSA</th>
<th>Molecular weight (g/mol)</th>
<th>fa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>4.5</td>
<td>4.27</td>
<td>65.26</td>
<td>275.85</td>
<td>4.23</td>
<td>357.80</td>
<td>100</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2.97</td>
<td>13.4</td>
<td>53.15</td>
<td>88.75</td>
<td>1.67</td>
<td>138.12</td>
<td>100</td>
</tr>
</tbody>
</table>

The molecular surface is described by the polar surface area (PSA) and the non-polar surface area (NPSA).

2.4. Concentrations

To avoid toxicity [as reflected by decreased monolayer integrity, using mannitol and transepithelial electrical resistance (TER) as markers] and potential solubility problems, all drugs were used at low concentrations. In particular, since it has been reported that indomethacin concentrations of ≥500 μM can damage the Caco-2 cell monolayer and thereby affect TER and the paracellular pathway (Tang et al., 1993), we decided to investigate indomethacin at 5, 50 and 100 μM. Salicylic acid was used at a concentration of 25 μM, except in the concentration-dependency study, where a concentration range of 25 μM to 33 mM was used.

2.5. Cell culture

Caco-2 cells (passages 27–41) were cultivated as previously described (Neuhoff et al., 2003), i.e. identical source, passage, culture medium, and seeding density.

2.6. Transport studies

Studies investigating the transport of indomethacin and salicylic acid across Caco-2 cell monolayers were performed as previously described (Neuhoff et al., 2003). Briefly, bidirectional (apical-to-basolateral: a–b; basolateral-to-apical: b–a) transport rates for the test compounds were measured across Caco-2 monolayer cultures grown in the Transwell® system (Cat. No. 3401, Corning Costar® Corporation, Cambridge, MA, USA) for 26 ± 1 days. The amount of drug transported was determined from the radioactivity content of the samples using a liquid scintillation counter (Wallac, Turku, Finland). The permeability of the monolayer to the paracellular marker mannitol was determined simultaneously in separate monolayers. The values for TER were measured at 37°C with an epithelial voltohmeter (EVOM), equipped with an SX-2 electrode (World Precision Instruments Inc., Sarasota, FL, USA). TER values of 280 ± 40 Ω cm<sup>2</sup> and permeability to mannitol of 0.36 ± 0.11 × 10<sup>-5</sup> cm s<sup>-1</sup> were used as integrity assurance (Neuhoff et al., 2003).

To investigate the dependence of the influx on temperature, the transport rate for salicylic acid was measured at 4, 7, 17, 27 and 37°C in transport buffer after preincubation of the cell monolayer for 30 min at the desired temperature. Q<sub>10</sub> values (the ratio of the transport rate at temperature T to that at T + 10 K) were between 1.4 and 2.9. In general, the Q<sub>10</sub> values fell as the temperature increased and the buffer pH decreased. Arrhenius plots of the logarithmic permeability values at the same donor concentration versus the reciprocal of the absolute temperature within one pH-gradient system were linear in the range of temperatures studied (r<sup>2</sup> > 0.95). There was no evidence for a phase change within this temperature range. Values for the activation energy (E<sub>a</sub>) were determined from the slope of the linear regression lines and were calculated in units of kJ mol<sup>-1</sup>.

For the inhibition studies, the cell monolayers were preincubated for 25 min in the presence of the inhibitor on both sides without the test compound. The inhibitors

---

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
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The molecular surface is described by the polar surface area (PSA) and the non-polar surface area (NPSA).
used included the general inhibitors DIDS (10 μM), which is a non-specific anion exchange inhibitor that modifies the lysine residues of membrane proteins, and phloretin (nominal concentration: 1 mM), which is a non-specific inhibitor of several active transport systems. Phloretin inhibits glucose transport in Caco-2 cells (Bissonnette et al., 1996) and is thus a potent indirect but reversible inhibitor of the proton-dependent MCT1 (Stein et al., 2000). L-Lactic acid (10 mM) was included as a more specific and enantioselective competitive inhibitor of MCT1 (Stein et al., 2000; Tamai et al., 1995), and α-L-acid (10 mM) was included as a non-substrate of MCT1. In addition, primazatin (5 mM), rifamycin SV (100 μM), and α-L-lactic acid (10 mM) were used as inhibitors of the proton-dependent OA TP-B. Inhibition of the apical proton-dependent transporter, PEPT1, was not performed, since neither indomethacin nor salicylic acid fulfilled the structural requirements as substrates for this transporter (Bailey et al., 2000; Gebauer et al., 2003).

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). The buffer was removed from both sides of the monolayers which were then incubated for 25 min in the presence of inhibitor at 37 °C with fresh buffer containing the test compound (at concentrations from 25 μM to 1 mM, depending on the compound; concentrations are given in the figure legend). The experiments involving indomethacin were always performed in parallel with those involving salicylic acid. Positive controls of transporter function, e.g., α-L-lactic acid for MCT1, were included in each experiment.

For instance, salicylic acid inhibited the a–b influx of the MCT1-specific substrate L-lactic acid in a concentration-dependent manner (apical pH 5.0, basolateral pH 7.4). 20 mM salicylic acid reduced the permeability of 25 μM L-lactic acid by 80% (from 10.83 ± 0.79 × 10−6 cm s⁻¹ to 2.21 ± 0.06 × 10−6 cm s⁻¹). Thus, the functional activity of apically localized MCT1 was confirmed (Tamai et al., 1995).

2.7. Data analysis

All experiments were performed under “sink” conditions, which allowed the apparent permeability coefficients (Papp) to be calculated as described previously (Artursson, 1990; Hunter et al., 1991). Mass balance was taken into account in order to correct for compound retention in the cell monolayer. The flux of all test compounds was linear over time under all conditions (r² ≥ 0.98). The ratio of a-to b-to a-transport was calculated to highlight any potential asymmetry. Thus, (apical) drug influx ratios were calculated using the following equation:

\[
\text{influx ratio}_{\text{apical}} = \frac{P_{\text{app}}(\text{a} \to \text{b})}{P_{\text{app}}(\text{b} \to \text{a})}\]

The mass balance (recovery, R) is the percentage of original drug mass accounted for at the end of the experiment (the sum of the amounts on the apical and basolateral sides). The mass balance was calculated using the following equation:

\[
\%R = \frac{[(C_{\text{a}(t)}V_{\text{a}}) + (C_{\text{b}(t)}V_{\text{b}})]100}{C_{\text{a}(0)}V_{\text{d}}}\]  

where C_{\text{a}(t)}, C_{\text{b}(t)}, and C_{\text{a}(0)} are the drug concentrations on the apical and basolateral sides of the monolayer at time t, V_{\text{a}} and V_{\text{b}} are the volumes of the apical and basolateral compartments, and V_{\text{d}} is the volume of the donor solution added to the appropriate side of the monolayer.

Values are expressed as the means of at least three measurements ± standard deviation (S.D.). The experiments were repeated at least twice. The statistical difference between the permeabilities of the cells to the drugs at different pH gradients or in the presence of inhibitors was calculated using an unpaired t-test with a two-tailed distribution for comparison of two mean values. ANOVA was used when more than two mean values were compared; p < 0.01 was considered statistically significant.

3. Results and discussion

3.1. pH-Dependent Caco-2 cell permeability to indomethacin

Transport experiments in Caco-2 cells are often conducted using a pH gradient in which the pH on the apical and basolateral sides is kept at 6.0–6.5 and 7.4, respectively (e.g. Sun et al., 2002; Yamashita et al., 2000; Yee, 1997). This approach attempts to mimic the acidic microclimate of the small intestine (Daniel et al., 1985; McEwan and Lucas, 1990). The transport rate for indomethacin at apical/basolateral pH 6.5/7.4 was compared with the rate obtained when the pH on both sides was equal: pH 7.4/7.4, i.e. in the absence of a pH gradient (since the intracellular pH is close to 7.4), and also with that obtained at pH 6.5/6.5, i.e. in the presence of one pH gradient over the apical membrane and a second pH gradient over the basolateral membrane of the cell.

At the physiological apical pH of 6.5, the transport rate was significantly higher in the absorptive (a–b) direction, giving an influx ratio of 6.4 ± 0.2 (Fig. 1B). In contrast, when the same pH was used in the apical and basolateral compartment, the permeability of the cells to indomethacin, although higher at pH 6.5 than at 7.4 (Fig. 1A), was essentially the same in either direction (Fig. 1). These results can be explained as follows. When the pH is lowered in the donor compartment, the fraction of uncharged drug is increased. This provides a greater driving force for transcellular diffusion of the uncharged species and, in the presence of a pH gradient, an asymmetric flux of the acidic drug. The same reasoning applies when the pH is lowered in the receiver compartment. However, when the pH is the same on both sides of the cell monolayer, the driving force for flux of the uncharged species...
will be the same and, consistent with the pH-partitioning theory, no net flux is observed.

To mimic all possible pH conditions in the intestinal tract in vivo, the apical pH was varied over the entire physiological pH range of the intestinal tract (pH 5.0–8.0), while the basolateral pH was kept constant at pH 7.4. The transport rate of indomethacin increased 14-fold (from $30 \pm 2 \times 10^{-6}$ to $414 \pm 14 \times 10^{-6}$ cm s$^{-1}$) in the a-b direction with decreasing pH in the apical chamber, while transport in the b-a direction decreased 20-fold (from $107 \pm 7 \times 10^{-6}$ to $5.2 \pm 0.4 \times 10^{-6}$ cm s$^{-1}$, Fig. 2). Together, these results indicate that the net influx of indomethacin is related to the amount of uncharged drug present in the donor compartment.

The assumption of a possible passive influx of acidic drugs across Caco-2 monolayers can be challenged by the fact that several active transport processes for acidic drugs require proton gradients such as those applied above. To address this issue, we also studied the concentration and temperature dependence of indomethacin transport since active, carrier-mediated transport is assumed to be affected by these parameters (Hidalgo and Borchardt, 1990). We also studied the transport of indomethacin in the presence of general and more specific carrier inhibitors.

### 3.2. Effects of concentration change

The bidirectional transport of indomethacin was independent of indomethacin concentration under all of the applied experimental conditions (data not shown). However, interpretation of the transport data may have been biased by another finding. When the pH in the donor and acceptor chambers was reduced to values below 6.5, a gradual pH- and concentration-dependent loss of mass balance was observed, resulting in a decrease in the observed permeability coefficients (Fig. 3). Importantly, when the loss in mass balance was accounted for, the permeability coefficients returned to the normal range (Fig. 3C). Since indomethacin is soluble under the experimental conditions and since no significant non-specific adsorption of indomethacin to the plastic wells was found at 37°C, we tentatively conclude that the compound was accumulated in the cell monolayer, due to the maintenance of the pH-gradient for instance by apical and basolateral localized sodium-proton exchangers (NHE) (Cavet et al., 2001). It has been reported that accumulation of salicylic acid in liposomes could be affected by the accumulation of another acid (Takagi et al., 1998), i.e. indomethacin. To examine, if a similar mechanism occurs in the Caco-2 cells, we investigated the effect of indomethacin on salicylic acid transport. However, the bidirectional transport of salicylic acid at an initial donor concentration of 25 μM was not affected by indomethacin (100 μM), indicating that indomethacin did not interfere with the salicylic acid transport (Fig. 8).

#### 3.2.1. Effects of temperature change

The studies of temperature dependence allowed calculation of the $E_a$ values for indomethacin transport using different pH-gradient systems. $E_a$ values between 30 and 107 kJ mol$^{-1}$ have previously been used to indicate active transport (Hidalgo and Borchardt, 1990). $E_a$ values for indomethacin transport in the absence and presence of pH gradients across the cell monolayers were corrected for changes in mass balance when necessary. The changes in $E_a$ were pH-dependent and therefore support the pH-partition theory.
Fig. 3. Bidirectional pH-dependent transport across Caco-2 cell monolayers of indomethacin at concentrations of 5, 50 or 100 μM. The buffer pH was the same on both sides of the monolayer (pH 5.0 or 7.4). (A) The apparent permeability coefficient ($P_{app}$) in the apical-to-basolateral direction (black bars) and the $P_{app}$ in the basolateral-to-apical direction (white bars) were equal at the buffer pH of 7.4. However, at lower buffer pH values (5.0), a reduced concentration-dependent transport rate was observed in both transport directions. (B) The corresponding mass balance values decreased with decreased pH and concentration. (C) The $P_{app}$ values after accounting for the mass loss reveal no concentration-dependent transport in any transport direction. Each value indicates mean ± S.D. ($n ≥ 4$).

However, a significant overlap between the $E_a$ values for indomethacin (passively) and actively transported compounds was observed ($E_a$ values for indomethacin ranged from 35 to 48 kJ mol$^{-1}$), precluding their use as a reliable indicator of active transport (Table 2).

### 3.2.2. Inhibition of active transport

The general (non-specific) inhibitors DIDS and phloretin had no effect on the indomethacin transport rate (data not shown). Nor did the more specific inhibitor of MCT1, $L$-lactic acid or its enantiomer $D$-lactic acid. Likewise, inhibition of OA TP-B with pravastatin, BSP or rifamycin SV had no significant effect, despite high mRNA levels for OA TP-B in the Caco-2 cells used in this study (Seithel et al., 2004).

Table 2: Activation energy ($E_a$) values for indomethacin and salicylic acid

<table>
<thead>
<tr>
<th>Apical pH</th>
<th>Basolateral pH</th>
<th>$E_a$ (indomethacin) (kJ mol$^{-1}$)</th>
<th>$E_a$ (25 μM salicylic acid) (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0/7.4</td>
<td></td>
<td>35.0</td>
<td>50.0</td>
</tr>
<tr>
<td>5.5/7.4</td>
<td></td>
<td>38.0</td>
<td>54.3</td>
</tr>
<tr>
<td>6.0/7.4</td>
<td></td>
<td>n. d.</td>
<td>58.7*</td>
</tr>
<tr>
<td>6.5/7.4</td>
<td></td>
<td>n. d.</td>
<td>69.1</td>
</tr>
<tr>
<td>7.4/7.4</td>
<td></td>
<td>48.3</td>
<td>69.8</td>
</tr>
<tr>
<td>5.0/5.0</td>
<td></td>
<td>35.0</td>
<td>37.9</td>
</tr>
<tr>
<td>5.5/5.5</td>
<td></td>
<td>28.0</td>
<td>42.1</td>
</tr>
<tr>
<td>6.5/6.5</td>
<td></td>
<td>n. d.</td>
<td>64.7</td>
</tr>
</tbody>
</table>

n.d.: not determined.

* Estimated value.

Indomethacin is frequently used as an allosteric inhibitor of several adenosine triphosphate binding cassette (ABC)-transporters without being itself a substrate of these efflux systems (Draper et al., 1997). Indomethacin also interacts with several members of the OCT and OAT families, and may have an allosteric interaction with the OCTs (Khamdang et al., 2002). The function of these transporters, however, is not proton-gradient dependent and, since concentration-dependent transport was not seen under any of the studied conditions (see above), it can be inferred that indomethacin is not actively transported by any of these transporters. This is not surprising, since indomethacin is only a weak substrate for MCT1 and OA TP-B transporters, further supporting the hypothesis that it is passively transported across the human intestine.

### 3.3. pH-Dependent Caco-2 cell permeability to salicylic acid

No significant difference in the bidirectional transport of salicylic acid was observed with equal apical and basolateral pH values (pH 7.4 or 6.5; Fig. 4) although, as seen with indomethacin, the permeability of the cells to salicylic acid increased with decreased buffer pH. As also seen with indomethacin, the $a$–$b$ transport rates were increased and the $b$–$a$ transport rates were decreased when cells were exposed to the apical pH-gradient, mimicking the physiological acidic microclimate (apical pH 6.5; basolateral pH 7.4), resulting in an influx ratio of 5.0 ± 0.2 (Fig. 4B). When the apical pH was decreased to 5.0 (apical pH 5.0; basolateral pH 7.4), the ratio of $a$–$b$ permeability to $b$–$a$ permeability reached a maximum of 140 (Fig. 5). This dramatic increase was caused by a pronounced (90-fold) increase in $a$–$b$ transport (from $7.8 ± 0.3 \times 10^{-6}$ to $704 ± 16 \times 10^{-6}$ cm s$^{-1}$) and a five-fold decrease in $b$–$a$ transport (from $23 ± 2 \times 10^{-6}$ to $5.2 ± 0.3 \times 10^{-6}$ cm s$^{-1}$; Fig. 5). All these results were in agreement with those obtained for the passively transported acidic drug, indomethacin (Figs. 1 and 2). However, since...
salicylic acid undergoes carrier-mediated proton-dependent transport (Takanaga et al., 1994), the results also show that bidirectional transport experiments cannot distinguish active proton-dependent transport from passive diffusion for acidic compounds.

3.3.1. Effects of concentration change

While the transport of salicylic acid did not appear to be dependent on concentration in the absence of a pH gradient (apical and basolateral pH 7.4, data not shown), there was clear concentration dependence with physiologically relevant pH gradients (acidic apical pH < 7.4; basolateral pH 7.4; Fig. 6). Thus, when the concentration of salicylic acid was increased from 25 μM to 33 mM, the permeability was reduced by 80%. The calculated pH-dependent apparent $K_m$ value of 5.4 ± 0.7 mM (apical pH 5.0; basolateral pH 7.4) is in agreement with published $K_m$ values for salicylic acid transport in Caco-2 cells (Takanaga et al., 1994). These results indicate that salicylic acid displayed both pH-gradient dependent active transport and passive diffusion. In contrast to our results for indomethacin, the mass balance of salicylic acid was not affected, and significant trapping of salicylic acid in the cytosol can, therefore, be excluded. We speculate that salicylic acid is effluxed from the cell monolayer by a basolateral transporter, such as the heterodimeric organic solute transporter (OST)α/β for which mRNA was recently detected in kidney and intestine (M. L. Hubbert et al., Falk Symposium No 141, Stockholm, Sweden, abstract No 52, 2004; http://www.falkfoundation.com/pdf/FS141-Abstractband-Internet.pdf). A role of a basolateral transporter (e.g. OSTα/β) could also explain the findings of Takagi and co-worker, who studied pH-dependent, carrier-independent uptake of salicylic acid and some other monocarboxylic acidic compounds in liposomes consisting of egg yolk phosphatidylcholine. These investigators found significant accumulation of salicylic acid in the liposome system, which lacks the OSTα/β transporter (Takagi et al., 1998). In contrast to pure passive diffusion, this passive influx process demonstrated saturation and competitive inhibition phenomena, due to the accumulation of compound in the liposomes.

In order to investigate whether active transport requires a proton gradient over the apical membrane only or whether it is needed over the whole monolayer (both apical and basolateral membrane), the dependency of the transport rates on concentration in an apical pH-gradient system (apical pH
suggests that cell suspensions which mimic only one pH gradient, some models, will not reflect the in vivo situation. This also proteins, such as brush border membrane vesicles and liposome models, may not always reflect the in vivo situation for compounds like salicylic acid.

5.0; basolateral pH 7.4; Fig. 6) was compared with that in a system with equal donor and receiver buffer pH values that were lower than pH 7.4. Under the latter conditions, the bidirectional transport rates were significantly and equally reduced compared with the corresponding apical pH-gradient system (Fig. 7). It is therefore suggested that, to mediate transepithelial transport, the relevant carrier-mediated transporters require a proton gradient over the entire cell, i.e. apical pH < 7.4 and basolateral pH 7.4, rather than between the extracellular and intracellular compartments only, i.e. apical pH < 7.4 equal to the basolateral pH < 7.4. These results also suggest that additional proteins in the basolateral membrane (i.e. those that maintain a proton gradient over the entire cell like the NHE1 (Cavet et al., 2001), or those that may accept a proton gradient over one membrane, i.e. the difference between buffer and intracellular pH, may not always reflect the in vivo situation for compounds like salicylic acid.

### 3.3.2. Effects of temperature change

As expected, the transport of salicylic acid (25 μM) was temperature dependent. The highest $E_a$ of 70 kJ mol$^{-1}$ was obtained in the absence of a pH gradient (apical and basolateral pH 7.4), i.e. under conditions without proton-dependent active transport of salicylic acid (Table 2). The $E_a$ decreased to 38 kJ mol$^{-1}$ when the pH was lowered to 5.0 on both sides of the monolayer. Intermediate $E_a$ values were obtained in the more physiological pH-gradient systems. Takanaga and co-workers reported the temperature dependency of salicylic acid, with an $E_a$ of 54 kJ mol$^{-1}$ (Takanaga et al., 1994). They determined the $E_a$ for the transcellular transport of salicylic acid (apical pH 6.0; basolateral pH 7.3) from the rates across Caco-2 cell monolayers at 37 and 4°C. For the purposes of comparison, the $E_a$ at an apical/basolateral pH gradient of 6.0/7.4 was therefore estimated from the Arrhenius plots of all investigated apical pH-gradient systems. An extrapolated $E_a$ of 58.7 kJ mol$^{-1}$ was obtained, which is in good agreement with Takanaga’s published results (Takanaga et al., 1994). Thus, as for indomethacin, it was difficult to distinguish active from passive transport of salicylic acid using $E_a$ values, since temperature dependency is not necessarily correlated with carrier-mediated transport and $E_a$ values are pH-dependent.

### 3.3.3. Inhibition of active transport

Phloretin only affected the a–b transport of salicylic acid in the apical pH-gradient system (Fig. 7A). It had no effect on salicylic acid flux when the same pH was applied on both sides of the monolayers. Transport rates in the b–a direction were not affected at all (Fig. 7B). These data suggest that salicylic acid transport is affected mainly by proton-dependent apical influx (or basolateral efflux) systems.

It has been suggested that MCT1, which is abundantly expressed in the small intestine, is involved in the transport of monocarboxylic acids like salicylic acid (Tamai et al., 1995) and that MCT1-expressing Caco-2 cells are suitable for in vitro studies of this transporter (Tamai et al., 1995). In our studies, salicylic acid transport was inhibited by L-lactic acid, pravastatin and their combination, but not by D-lactic acid, BAF, rifamycin SV or DIDS (Fig. 8). The stereoselectivity for lactic acid was expected for MCT1 inhibition (Tamai et al., 1995). The substrate specificity of the MCT transporter is very broad and overlaps with that of the OAT family (Halestrap and Price, 1999; Sekine et al., 2000). Therefore it was not surprising that pravastatin, an inhibitor of OATP-B, also inhibited salicylic acid transport. According to our inhibition studies, MCT1 is only responsible for about 10–15% of the total carrier-mediated transport of salicylic acid and OATP-B is responsible for at least an additional 10–15% (Fig. 8). The combination of pravastatin and L-lactic acid resulted in an additive decrease in salicylic acid transport.
In a previous study in human erythrocytes, the transport of salicylic acid was ascribed to two parallel processes, one involving passive diffusion of the un-ionized species. Studies of concentration dependence and use of transporter inhibitors gave more reliable results. Since an acidic microclimate is found adjacent to the enterocytes in a large part of the rat and human small intestine, our in vivo observations of the pH-dependent passive and active transport processes can be extended to the in vitro situation. We therefore conclude that experimental conditions that reflect the acidic microclimate in Caco-2 cells will give a better reflection of the permeability of the intestine to acidic drugs in vivo, independently of the relevant active transport process.

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