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pH-Dependent and Carrier-mediated Transport of Salicylic Acid Across Caco-2 Cells

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Abstract—The transport of monocarboxylic acid drugs such as salicylic acid was examined in the human colon adenocarcinoma cell line, Caco-2 cells that possess intestinal epithelia-like properties. [^{14}C]Salicylic acid transport was pH-dependent and appeared to follow the pH-partition hypothesis. However, 10 mM unlabelled salicylic acid significantly reduced the permeability coefficient of [^{14}C]salicylic acid. Kinetic analysis of the concentration dependence of the permeation rate of salicylic acid across Caco-2 cells showed both saturable ($K_t = 5{\cdot}28 \pm 0{\cdot}72$ mM $J_{max} = 36{\cdot}6 \pm 3{\cdot}54$ nmol min^{-1} (mg protein)$^{-1}$) and non-saturable ($k_d = 0{\cdot}37 \pm 0{\cdot}08$ μL min^{-1} (mg protein)$^{-1}$) processes. The permeation rate of [^{14}C]salicylic acid was competitively inhibited by both acetic acid and benzoic acid, which were demonstrated in our previous studies to be transported in the carrier-mediated-transport mechanism which is responsible for monocarboxylic acids. Furthermore, certain monocarboxylic acids significantly inhibited [^{14}C]salicylic acid transport, whereas salicylamide and dicarboxylic acids such as succinic acid did not. From these results, it was concluded that the transcellular transport of [^{14}C]salicylic acid across Caco-2 cells is by the pH-dependent and carrier-mediated transport mechanism specific for monocarboxylic acids.

Foreign organic monocarboxylates have been widely accepted to be absorbed from the intestine by passive diffusion, depending only on the degree of protonation of the carboxylate moieties and the lipid solubility of the unionized molecules. However, deviations from the general rule of non-ionic diffusion i.e. the pH-partition hypothesis, have been observed, for example, in the absorption of benzoic acid and salicylic acid (Brodie & Hogben 1957; Nogami & Matsuzawa 1961), both of which are rapidly absorbed from the small intestine despite almost complete dissociation at the pH of the small intestinal lumen. Various explanations have been applied for such deviations from the pH-partition hypothesis; including existence of the virtual pH (Brodie & Hogben 1957), the microclimate pH at the mucosal surface (Said et al 1986; Schanker et al 1958), and absorption of the ionized form through the paracellular pathway (Nogami & Matsuzawa 1961). Although all of these explanations are based on intestinal absorption by passive diffusion mechanisms, some previous results obtained by using intestinal perfusion methods suggest absorption of salicylic acid by a carrier-mediated mechanism (Fisher 1981; Takahata et al 1986).

In our previous paper, we demonstrated that monocarboxylic acids such as acetic acid and nicotinic acid are transported across the intestinal brush-border membrane to a major extent by carrier-mediated mechanisms including proton-cotransport and pH-dependent anion exchange systems (Tsuji et al 1990; Simanjuntak et al 1990, 1991). Furthermore, by using monolayers of human colon adenocarcinoma cell line, Caco-2 cells, we demonstrated that benzoic acid was transported via the carrier-mediated mechanism specific for monocarboxylic acids and the transport was energized by the inwardly directed proton gradient across the apical membrane (Tsuji et al 1994). Salicylic acid inhibited benzoic acid transport competitively and displayed a counter-transport effect on the uptake of [^{14}C]benzoic acid, suggesting that salicylic acid is also transported via the common carrier with benzoic acid. These observations suggest that the deviation of the absorption of weak organic acids from the pH-partition hypothesis is interpretable by the carrier-mediated transport mechanism rather than by another passive diffusion model. Therefore, to confirm the participation of a carrier-mediated mechanism in salicylic acid transport, we investigated the transcellular transport of salicylic acid across Caco-2 cells. This experimental system has merit in that it provided an intestinal epithelial model consisting of a highly differentiated cell monolayer when grown on a microporous polycarbonate membrane (Hidalgo et al 1989). Furthermore, since Caco-2 cells have been shown to have the function of the small intestine (Hidalgo et al 1989), this polarized epithelial cell line is useful in the study of the transcellular transport mechanism of salicylic acid.

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Materials and Methods

Materials

The Caco-2 cell line was obtained from Eisai Co., Ltd (Tokyo, Japan) and was confirmed mycoplasma-negative using the Hoechst 33258 test (Flow laboratories Ltd, Irvine, UK). Dulbecco's Modified Eagle's Medium, foetal calf serum, and non-essential amino acids were obtained from Gibco (Grand Island, NY, USA). L-Glutamine, penicillin G, and streptomycin were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Polycarbonate membrane, Transwell clusters, 11·2 mm in diameter and 3·0 μm pore size were purchased from Costar (Bedford, MA, USA). [^{14}C]Salicylic acid (2·1 GBq mmol^{-1}) and [^{3}H]mannitol (1110 GBq mmol^{-1}) were purchased from New England Nuclear (Boston, MA, USA). All other chemicals were of reagent grade and were obtained commercially.

Cell culture and transport experiments

The method for preparation of confluent monolayers of Caco-2 cells on Transwell has been described in detail previously (Tsuji et al 1994). All cells used in this study were between passages 55 and 60.

Transport experiments were performed by using Caco-2 cells cultured for 21–23 days on Transwell as described previously (Tsuji et al 1994). The basolateral side (receiver side) was filled with 1·5 mL Hanks balanced salt solution (HBSS) containing (mM): $CaCl_2$ 0·952, KCl 5·36, KH_2PO_4 0·441, $MgSO_4$ 0·812, NaCl 136·7, Na_2HPO_4 0·385, D-glucose 25 and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) 10 (pH 7·3). To initiate the transport experiments, 0·5 mL of HBSS containing [^{14}C]salicylic acid at the desired pH was loaded onto the apical side (donor side) of the cell. The pH of the donor side was adjusted to the desired pH value by 2-(*N*-morpholino) ethanesulphonic acid (Mes) or HEPES. At an appropriate time a 500 μL sample was taken from the receiver side and replaced with an equal volume of fresh HBSS.

Analytical method

The amount of salicylic acid transported was estimated from the radioactivity in the sample. Radioactivity was determined using a liquid scintillation counter (LSC-1,000, Aloka Co. Ltd, Tokyo, Japan). Cellular protein was measured by the method of Lowry et al (1951) by using bovine serum albumin as a standard.

The transepithelial electrical resistance (TEER) was measured using Millicell-ERS (Millipore, Bedford, MA, USA). The Caco-2 cells were exposed to HBSS for 10 min at 37°C before each measurement. The TEER value of control monolayers was over 300 Ω cm^2.

Data analysis

The permeation rate (nmol min^{-1} (mg protein)$^{-1}$), J, was evaluated from the slope of the initial linear portion of plots of the amount transported (nmol min^{-1} (mg protein)$^{-1}$) against time (min), calculated by linear regression analysis. The permeation coefficient (μL min^{-1} (mg protein)$^{-1}$) was obtained by dividing the permeation rate by the drug concentration (nM) at the apical side.

The kinetic parameters for the saturable transport across Caco-2 cells were estimated by fitting equation 1 using the nonlinear least-square regression analysis program, MULTI (Yamaoka et al 1981):

$$J = \frac{J_{max}C}{K_t + C} + k_dC \quad (1)$$

where C is the drug concentration at the apical side, J_{max} is the maximum permeation rate, K_t is the Michaelis–Menten constant and k_d is the non-saturable permeation rate. All data are expressed as mean ± s.e.m. Statistical analysis was performed by using Student's two-tailed *t*-test. The level of significance was taken as $P < 0.05$.

Results and Discussion

Fig. 1 shows time courses for the transport of [^{14}C]salicylic acid from apical to basolateral side at acidic or neutral apical pH (6·0 and 7·3) across Caco-2 cells. Although

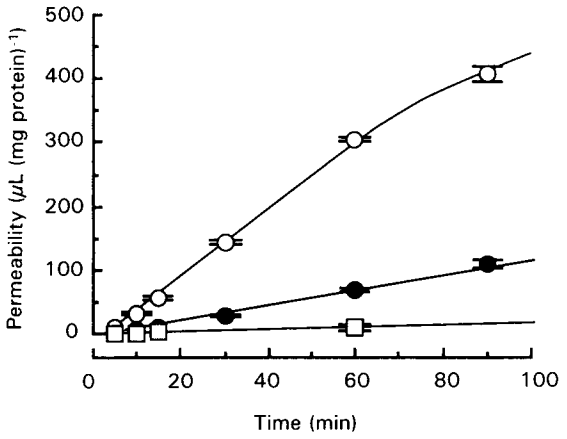

FIG. 1. Time-courses for the transport of [^{14}C]salicylic acid and [^{3}H]mannitol across Caco-2 cells. Permeability of [^{14}C]salicylic acid (5 μM) or [^{3}H]mannitol (33 nM) was measured at 37°C by incubating Caco-2 cells in HBSS buffer at apical pH 6·0 (○) or 7·3 (●) and at basolateral pH 7·3. Permeability of [^{3}H]mannitol was measured on the apical side pH 6·0 (□). Each point represents the mean ± s.e.m. of three experiments.

salicylic acid was reported to increase the permeability of the intestinal barrier by chelation of divalent metal ions (Kunze et al 1972), the transport of [^{3}H]mannitol, which represents paracellular transport feasibility, did not change in the presence of salicylic acid. Therefore, salicylic acid apparently has no effect on the membrane integrity and the permeability coefficient of [^{14}C]salicylic acid is ascribed to transcellular transport not to paracellular transport. Moreover, since no catabolism of salicylic acid was observed during the transport across Caco-2 cells as assessed by HPLC analysis of transported sample in the receiver side (data not shown), the transport can be referred to the permeation of the intact molecule.

Since the proton gradient was suggested to be a driving force of benzoic acid transport across Caco-2 cells as reported previously (Tsuji et al 1994), the effect of apical pH on the transcellular transport of [^{14}C]salicylic acid was examined in the pH range from 5·0 to 7·3 (Fig. 2). At the constant basolateral pH of 7·3 which is close to the intracellular pH of Caco-2 cells (Dantzig & Bergin 1990), the permeability coefficient of [^{14}C]salicylic acid markedly increased with decreasing pH of the apical side. Since the permeability coefficient of mannitol was not affected by the change of apical pH, the pH-dependence of the permeability coefficient of salicylic acid is not ascribed to the non-specific effect of pH such as alterations of the membrane integrity or the use of a paracellular pathway. In the presence of 10 mM unlabelled salicylic acid, the transcellular transport of [^{14}C]salicylic acid was significantly reduced. Furthermore, the extent of inhibitory effect of unlabelled salicylic acid increased with lowering pH of the apical side. These results suggest that a pH-dependent and carrier-mediated mechanism contributes to the transport of salicylic acid, especially at acidic pH.

One important criterion for the carrier-mediated transport is saturation of the permeation rate; therefore, the effect of increasing concentrations of salicylic acid on the permeation rate across Caco-2 cells was studied. As shown in Fig. 3, the

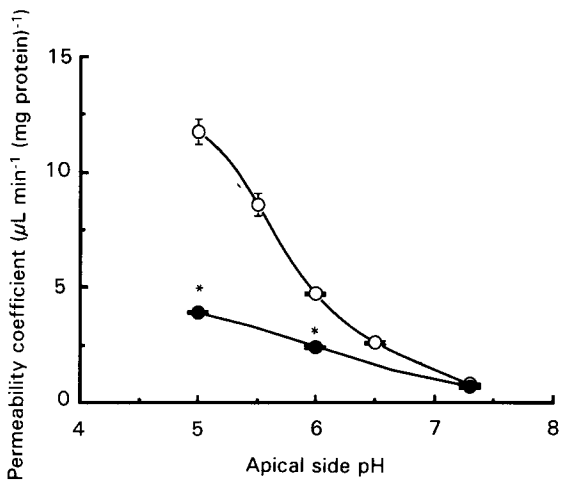

FIG. 2. pH dependence of [^{14}C]salicylic acid transport across Caco-2 cells. Permeability coefficient of [^{14}C]salicylic acid was measured at 37°C by incubating Caco-2 cells in HBSS buffer in the absence (○) or presence (●) of unlabelled salicylic acid (10 mM). Each point represents the mean ± s.e.m. of three experiments. *$P < 0.05$ compared with the permeability coefficient measured at the same apical side pH without unlabelled salicylic acid.

permeation rate of salicylic acid was saturable and the kinetic parameters obtained by fitting equation 1 were maximum permeation rate, J_{max} 36·6 ± 3·54 nmol min^{-1} (mg protein)$^{-1}$; Michaelis–Menten constant, K_t 5·28 ± 0·72 mM; non-saturable first-order rate constant, k_d 0·37 ± 0·08 μL min^{-1} (mg protein)$^{-1}$. Although the finding of a proportional relation between the luminal concentrations and rate of salicylic acid transport has been reported by Schanker et al (1958), in our study, the permeation rate of salicylic acid declined as the luminal concentration rose above 5 mM. It should be noted, however, that Schanker et al (1958) used an in-vivo model with a significant aqueous resistance across the diffusion boundary layer. The finding that at high apical concentrations the permeation rate was reduced is not explicable by supposing that salicylic acid is absorbed by passive diffusion, because no significant change in the integrity of monolayers of Caco-2 cells was observed by exposure at 40 mM salicylic acid during the experimental period (15 min) when assessed by the permeability coefficient of [^{3}H]mannitol and TEER (data not shown). Furthermore, the transcellular transport of salicylic acid was shown to be remarkably temperature-dependent (Fig. 3). The activation energy (E_a) for transcellular transport evaluated from the rates at 37 and 4°C was about 54 kJ mol^{-1}. These concentration and temperature dependencies in the transport indicate that the transcellular transport of salicylic acid is performed by a carrier-mediated mechanism.

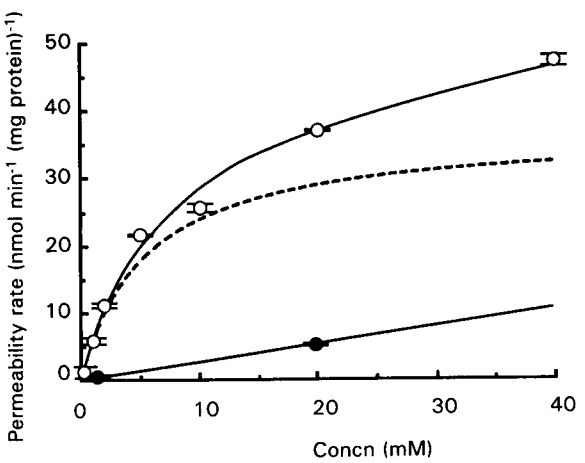

FIG. 3. Concentration and temperature dependencies of [^{14}C]salicylic acid transport across Caco-2 cells. Permeation rate of salicylic acid was measured at 37 (○) or 4°C (●). The incubation conditions were identical to those described in the legend to Fig. 1. The broken line represents the permeability rate for the saturable component calculated from the kinetic parameters obtained as mentioned in the text. Each point represents the mean ± s.e.m. of three experiments.

Table 1. Inhibitory effect of various compounds on salicylic acid permeability.

Inhibitor	Relative permeability (% of control)
Acetic acid	45·7 ± 1·74*
o-Anisic acid	29·4 ± 0·74*
Benzoic acid	25·6 ± 1·88*
m-Hydroxybenzoic acid	75·0 ± 1·14*
p-Hydroxybenzoic acid	58·4 ± 1·49*
Salicylic acid	36·7 ± 0·09*
Valproic acid	25·5 ± 1·23*
Salicylamide	94·0 ± 0·03
Succinic acid	96·9 ± 0·06

The permeability of [^{14}C]salicylic acid was measured at 37°C for 15 min by incubating Caco-2 cells in HBSS buffer (apical pH = 6·0, basolateral pH = 7·3) in the absence or presence of each inhibitor. Permeability coefficient of salicylic acid in the control study was 6·0 μL min^{-1} (mg protein)$^{-1}$. Concentrations of [^{14}C]salicylic acid and inhibitors were 5 μM and 10 mM, respectively. Each value represents the mean ± s.e.m. of 3–5 experiments. *$P < 0.05$.

To determine the structural specificity of the carrier-mediated transport system responsible for salicylic acid transport, the effects of various compounds on the permeability coefficient of salicylic acid were determined (Table 1). The result indicates that the permeability coefficient of [^{14}C]salicylic acid was significantly inhibited by all of the monocarboxylic acids tested, but not by succinic acid or salicylamide. These results demonstrate that the transporter for salicylic acid is specific for certain organic anions having one monocarboxylic acid moiety in the molecule. Among the organic anions having inhibitory effects, the effects of acetic acid and benzoic acid were further studied kinetically to determine whether they share a common binding site on the carrier protein. Fig. 4 shows the effects of acetic acid and benzoic acid on the permeation rates of salicylic acid by Lineweaver-Burk plots after the subtraction of the non-saturable component. The inhibitory constants, K_i, for acetic acid and benzoic acid were 8·56 ± 0·38 and 4·54 ± 0·57 mM, respectively. The inhibitory constant of benzoic acid is close to the K_t value (4·83 mM) obtained by the permeation rate of benzoic acid itself under the same condition across Caco-2 cells (Tsuji et al 1994). Furthermore, in our previous report, we showed that salicylic acid had a counter-transport effect on benzoic acid uptake by Caco-2 cells (Tsuji et al 1994), indicating that salicylic acid shares the transporter with benzoic acid.

Fig. 5 represents the relationship between the affinity of monocarboxylic acid drugs shown by $1/K_i$ to the salicylic acid transporter, estimated from the inhibitory effects

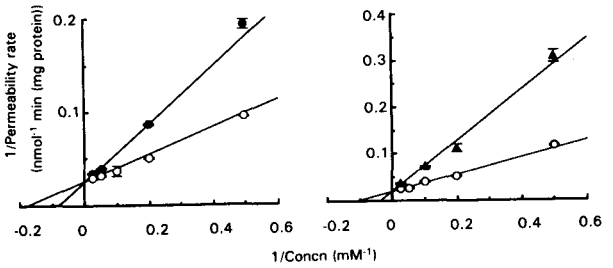

FIG. 4. Lineweaver-Burk plots for the transport of salicylic acid across Caco-2 cells. Permeability coefficient was measured in the absence (○) and the presence of 10 mM acetic acid (●) or benzoic acid (▲). The incubation conditions were identical to those described in the legend to Fig. 1. Each point represents the mean of three experiments.

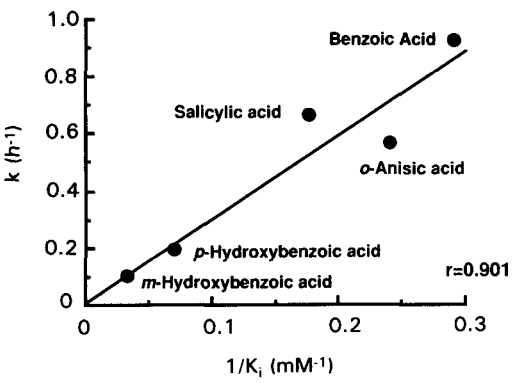

FIG. 5. Correlation between in-situ small intestinal absorption rate constants (k) and in-vitro affinity constants ($1/K_i$) of monocarboxylic acids. The values of k were obtained from Nogami et al (1968).

shown in Table 1 and in-situ small intestinal absorption rate constants obtained in rats (Nogami et al 1968), showing significant correlation ($r = 0.901$) between them. This result suggests that the intestinal absorption of these structural analogues is attributable to the common carrier-mediated mechanism demonstrated in the present study.

In conclusion, the overall results obtained in the present study indicate that salicylic acid is transported via a carrier-mediated transport mechanism specific for monocarboxylic acids across Caco-2 cells. Furthermore, in conjunction with the previous study of benzoic acid transport across Caco-2 cells (Tsuji et al 1994), we have demonstrated that a carrier-mediated transport system for monocarboxylic acid is functioning in Caco-2 cells with characteristics which seem to be in accordance with those of the carriers existing at the intestinal brush-border membrane. Therefore, the apparent pH-dependence of the intestinal absorption of salicylic acid should be ascribed to the same pH-dependent carrier-mediated mechanism, which is responsible for the enhanced permeability coefficient in the presence of an inwardly-directed H^+-gradient.

Acknowledgements

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