Carrier-Mediated Transport of Monocarboxylic Acids in Primary Cultured Epithelial Cells from Rabbit Oral Mucosa

Naoki Utoguchi,[1,2] Yoshiteru Watanabe,[1] Takahisa Suzuki,[1] Junko Maehara,[1] Yoshiaki Matsumoto,[1] and Mitsuo Matsumoto[1]

Received October 28, 1996; accepted December 24, 1996

Purpose. Using primary cultured rabbit oral mucosal epithelial cells (ROEpi), we investigated whether carrier-mediated drug absorption via the oral mucosal route occurs.

Methods. Oral mucosal epithelial cells were isolated from rabbit buccal mucosa and cultured on tissue culture plates. When the cells reached confluence, drug uptake experiments were performed. [^{14}C]Benzoic acid or [^{14}C]acetic acid was used as a marker for monocarboxylic acid carrier-mediated transport.

Results. The uptake of [^{14}C]benzoic acid by ROEpi occurred at a much lower rate at 4°C than at 37°C. The metabolic inhibitors, sodium azide and 2,4-dinitrophenol, significantly inhibited the uptake of [^{14}C]benzoic acid by ROEpi. Various monocarboxylic acids inhibited the uptake of [^{14}C]benzoic acid or [^{14}C]acetic acid by ROEpi, whereas dicarboxylic acids did not affect the uptake. Kinetic analysis using Lineweaver-Burk plots revealed that acetic acid competitively inhibited the uptake of [^{14}C]benzoic acid, and benzoic acid competitively inhibited the uptake of [^{14}C]acetic acid by ROEpi.

Conclusions. There exists a carrier-mediated transport system for monocarboxylic acids in oral mucosal epithelial cells.

KEY WORDS: oral mucosa; carrier-mediated transport; epithelial cells; monocarboxylic acid.

INTRODUCTION

The oral mucosal route is advantageous for drug delivery to the systemic circulation because exposure of the administered drug are prevented to gastrointestinal juices and its hepatic first-pass elimination (1,2). In general, the absorption of drugs from the oral mucosa can be explained in terms of the pH-partition hypothesis which is well illustrated by passive diffusion mechanisms (3–5). The hypothesis proposes that the rate of drug absorption depends on the percentage of drug molecules ionized and the lipid solubility of nonionized drug molecules.

Many carrier-mediated drug transport mechanisms in the intestinal epithelium have been studied using brush-border membrane vesicle techniques and cultured cells of the intestinal epithelium (6,7). Some monocarboxylic acids, dipeptides, and amino acids were shown to be transported by specific transporters in the intestinal epithelium. In a cultured monolayer of oral mucosal epithelial cells, fluorescence-labeled dextran is transported via a paracellular route (8) and β-adrenergic antagonist transport is dependent on a high apparent octanol/buffer distribution coefficient (9). However, no reports concerning carrier-mediated transport mechanisms of the oral mucosal epithelium have been published. In this present study, we investigated whether carrier-mediated drug absorption via the oral mucosal route occurs. We focused on the monocarboxylic acid transporter which is the most well-studied transporter in the intestinal epithelium. If the oral mucosal epithelium possesses monocarboxylic acid transporter, monocarboxylic acids, in the buccal dosage form, should be transported via this transporter. In this study, using primary cultured rabbit oral mucosal epithelial cells (ROEpi) and [^{14}C]benzoic acid or [^{14}C]acetic acid, we investigated whether carrier-mediated monocarboxylic acid transport via the oral mucosal route occurs.

MATERIALS AND METHODS

Materials

[^{14}C]Benzoic acid (54 Ci/mol) and [^{14}C]acetic acid (53 Ci/mol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]Mannitol (15–30 Ci/mmol) was purchased from New England Nuclear (Boston, MA). MCDB 153, epidermal growth factor, and trypsin type III were obtained from Sigma Chemicals (St. Louis, MO). Fetal calf serum (FCS) was purchased from Biotech International Ltd. (Australia). Dispase II was obtained from Boehringer Mannheim (Mannheim, Germany). Tissue culture plates were purchased from Costar (Cambridge, MA). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes) and 2-morpholinoethanesulfonic acid, monohydrate (Mes) were purchased from Dojin (Kumamoto, Japan). All other chemicals were of the purest available analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan)

Cell Culture

Oral mucosal epithelial cells were isolated from rabbit buccal mucosa and cultured using the method of Oda and Watson, with slight modifications (10). Briefly, male rabbits (Japan White, weight range: 3.0 to 3.5 kg) were sacrificed by administration of sodium pentobarbital and their buccae were excised, washed with phosphate-buffered saline (PBS) containing antibiotics (penicillin G, 200 U/ml; streptomycin, 200 μg/ml; and gentamicin, 40 μg/ml), and then cut with razors into small pieces (each about 3×6 mm in size). The tissue pieces were then incubated at 4°C in dispase II solution (2.4 U/ml) containing antibiotics. After a 24 h incubation, epithelial sheets were separated using a forceps and incubated at 37°C with 0.05% trypsin type III for 15 min after which the cells were suspended in PBS and then centrifuged for 5 min at 200×g. The cell pellets were suspended in a low-Ca^{2+} medium, MCDB 153, containing 10% FCS, insulin (5 μg/ml), transferrin (10 μg/ml), phosphorylethanolamine (14.1 μg/ml), penicillin G (100 U/ml), streptomycin (100μg/ml), gentamicin (20μg/ml), and epidermal growth factor (10 ng/ml), and the suspension was plated on collagen-coated 6- or 12-well tissue culture plates. After a 24h cultivation at 37°C in a 95% air-5% CO_2 humidified atmosphere, the cells were washed twice with PBS and fresh culture medium was added to each well. After 4 or 5 days of

[1] Department of Pharmaceutics, Showa College of Pharmaceutical Sciences, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194, Japan.

[2] To whom correspondence should be addressed.

0724-8741/97/0300-0320$12.50/0

cultivation, the cells had reached confluence, and drug uptake experiments were performed. The cell type was identified and the purity of the cell preparation was determined using an epithelial cell marker enzyme (alkaline phosphatase and leucine aminopeptidase) assay (8) and by indirect immunofluorescence staining of keratin (11). The cultured cells exhibited high levels of alkaline phosphatase and leucine aminopeptidase activity: 461 IU/μg of cell protein and 15.6 IU/μg of cell protein, respectively. More than 95% of the cells were positively stained for keratin (data not shown).

Uptake Experiments

The ROEpi at confluence were washed twice with Hanks' balanced salt solution (HBSS; 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.812 mM $MgSO_4$, 25 mM D-glucose and 10 mM Hepes for adjustment to pH 7.0 or 7.5 or 10 mM Mes for adjustment to pHs <6.5), and then the test solution conatining [^{14}C]benzoic acid or [^{14}C]acetic acid was added. [^{14}C]Benzoic acid or [^{14}C]acetic acid (0.25 μCi/ml) was used as a marker for monocarboxylic acid carrier-mediated transport. The pH of the test solution was 6.0 except in the pH-dependent uptake experiment. After 30 s, the test solution was aspirated and the cells were washed with ice-cold HBSS four times. For quantitation of drug uptake, the cells were suspended in 0.5 N NaOH and the suspension was incubated at 37°C for 12 h at which point, a half volume of 1.0 N HCl was added. [^{3}H]Mannitol was used as a marker for the extracellular fluid that adhered to the ROEpi. Radioactivity was quantitated using a liquid scintillation counter (Aloka, LSC-5100). Cellular protein was quantified using a protein assay kit (Bio-Rad, CA) with bovine serum albumin as a standard. Details of the conditions for each experiment are given in the figure legends or table footnotes.

Treatment with Protease or Amino Acid-Modifying Agents

Papain (0.5 mg/ml) was activated at 4°C by incubation with 5 mM cysteine and 2 mM ethylenediaminetetraacetic acid (EDTA) for 15 min. ROEpi were incubated at 37°C for 30 min with this freshly activated papain solution. In the case of trypsin treatment, the cells were incubated at 37°C for 15 min with 0.01 mg/ml trypsin. *N*-ethylmaleimide (NEM) and dithiothreitol (DTT) were added to the ROEpi and incubated at 37°C for 10 min. Then each preincubation solution was removed and the ROEpi were washed three times with HBSS after which uptake experiments were performed.

Data Analysis

To estimate the values of the kinetic parameters of saturable uptake by ROEpi, the uptake rate (J) was fitted to the following equation, which consists of both saturable and nonsaturable linear terms, using a nonlinear least-squares regression analysis program (12):

$$J = J_{max} \times C / (Kt + C) + k \times C \quad (1)$$

where J_{max} is the maximum uptake rate for a carrier-mediated process, C is the benzoic acid concentration, Kt is the half-saturation concentration (Michaelis constant), and k is a first-order rate constant.

Statistical Analysis

All results were expressed as means ± standard deviation (SD). Statistical analysis between two groups was performed using Student's *t*-test, and one-way analysis of variance (ANOVA) was used for single and multiple comparisons. *P* values of 0.05 or less were considered to indicate a statistically significant difference.

RESULTS

Concentration and Temperature Dependences

The effect of incubation temperature on the uptake of [^{14}C]benzoic acid by the ROEpi was studied. The uptake occurred at a much lower rate at 4°C than at 37°C, as shown in Fig. 1. Figure 1 also shows the relationship between the initial rate of uptake of [^{14}C]benzoic acid and its concentration in the medium. The results indicate that the uptake of benzoic acid consists of two processes, a saturable process evident at low concentrations and an apparently nonsaturable process evident at high concentrations. The uptake processes were analyzed according to Eq. (1). The kinetic parameters calculated for benzoic acid uptake were 380 nmol/30 s/mg of protein for J_{max}, 1.2 mM for Kt, and 35 μl/30 s/mg of protein for k.

Energy Dependence

The effects of metabolic inhibitors on the uptake of [^{14}C]benzoic acid were studied to determine whether this uptake requires metabolic energy (Table 1). Sodium azide (10 mM), a respiratory chain inhibitor, or 2,4-dinitrophenol (1 mM), an uncoupler of oxidative phosphorylation, significantly inhibited the uptake of [^{14}C]benzoic acid by the ROEpi.

pH-Dependent Uptake

Figure 2 illustrates the effect of incubation buffer pH in the range from 5.0 to 7.5 on [^{14}C]benzoic acid uptake by ROEpi. The rate of [^{14}C]benzoic acid uptake decreased with increasing pH from an acidic to a neutral pH. Moreover, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 50 μM), a pro-

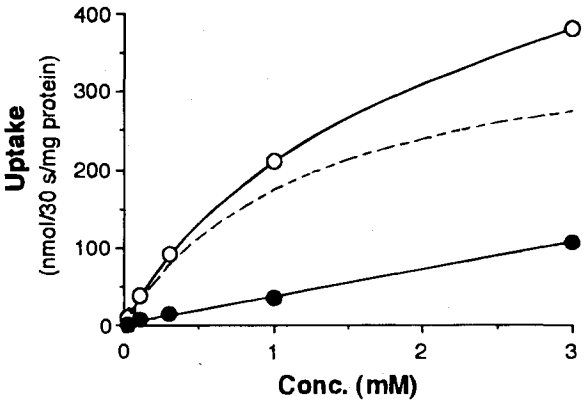

Fig. 1. Concentration and temperature dependence of [^{14}C]benzoic acid uptake by ROEpi. Uptake of [^{14}C]benzoic acid by ROEpi was measured at 37°C (○) or 4°C (●). The dotted line represents the uptake for the saturable component calculated from the kinetic parameters obtained as described in the text.

Table 1. Effects of Metabolic Inhibitors, Ionophores, or Anion-exchange Inhibitors on [^{14}C]Benzoic Acid Uptake by ROEpi

		Relative uptake (% of control)
Sodium azide	(10 mM)	14.2 ± 3.5[a]
2,4-Dinitrophenol	(1 mM)	16.0 ± 2.4[a]
FCCP	(50 μM)	12.2 ± 0.4[a]
DIDS	(100 μM)	91.7 ± 5.0

Note: ROEpi were pretreated with these agents. After a 15 min incubation, uptake experiments were performed.
[a] Significantly different from the control value ($P < 0.05$).

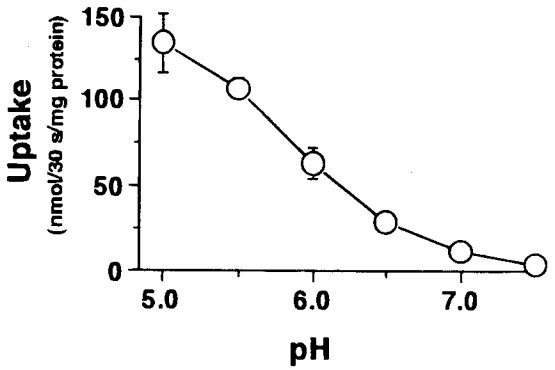

Fig. 2. pH Dependence of [^{14}C]benzoic acid uptake by ROEpi. Uptake of [^{14}C]benzoic acid by ROEpi incubated at 37°C in HBSS was measured. The vertical bar through each point represents the SD of four experiments.

tonophore, significantly inhibited the uptake (Table 1), whereas 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; 100 μM), an anion-exchange inhibitor, had no inhibitory effect. These findings suggest that [^{14}C]benzoic acid uptake by ROEpi is dependent on proton gradient and is carrier-mediated.

Specificity of the Carrier

To investigate the properties of the carrier involved in [^{14}C]benzoic acid uptake by ROEpi, we studied the effects of various mono- and dicarboxylic acids on this uptake (Table 2). Each monocarboxylic acid significantly inhibited the uptake of [^{14}C]benzoic acid, whereas none of the dicarboxylic acids had any significant effect on it. We also found that the ROEpi take up [^{14}C]acetic acid and that this uptake is significantly inhibited by monocarboxylic acids (Table 2). In contrast, dicarboxylic acids had no marked effect on the uptake of [^{14}C]acetic acid by ROEpi. These results suggest that the carrier which mediates monocarboxylic acid uptake by ROEpi is a nonspecific monocarboxylic acid carrier.

To study the mechanism of the benzoic acid or acetic acid uptake inhibition by the monocarboxylic acids, we analyzed the inhibitory effect kinetically. Figure 3A shows Lineweaver-Burk plots for the uptake of [^{14}C]benzoic acid by ROEpi in the absence or presence of 10 mM acetic acid. Acetic acid competitively inhibited the uptake of benzoic acid by the ROEpi. Figure 3B shows Lineweaver-Burk plots for the uptake of [^{14}C]acetic acid by ROEpi in the absence or presence of 10mM benzoic acid. Benzoic acid competitively inhibited the uptake of acetic acid by the ROEpi. The Kt for acetic acid was 2.2 mM and the inhibition constant of acetic acid was 2.5 mM on the ROEpi.

Effects of Proteases and Amino Acid-Modifying Agents

To further investigate the carrier-mediated benzoic acid uptake, additional experiments were performed to determine whether proteases and amino acid-modifying agents inhibit benzoic acid uptake by ROEpi (Table 3). The amount of [^{14}C]benzoic acid taken up by the ROEpi which were preincubated with papain was 25% of the control value. Trypsin also markedly inhibited the uptake, as did dithiothreitol (DTT), amino acid-modifying agents, and *N*-ethylmaleimide (NEM). These results suggest that a membrane protein is required for the uptake of benzoic acid by ROEpi and that the benzoic acid transporter requires histidyl and/or thiol residues for its function.

DISCUSSION

Many carrier-mediated transport systems in the small intestine have been investigated using brush-border membrane vesicles or cultured cells (Caco-2) (6,7). However, the existence of a carrier-mediated transport system in oral mucosal epithelium has not been previously reported. In the present study, we

Table 2. Effects of Various Carboxylic Acids on [^{14}C]Benzoic Acid or [^{14}C]Acetic Acid Uptake by ROEpi

		Relative uptake (% of control)	
		[^{14}C]Benzoic acid	[^{14}C]Acetic acid
Monocarboxylic acids	Acetic acid	54.6 ± 10.0[a]	N.D.
	Benzoic acid	N.D.	52.6 ± 5.2[a]
	Valproic acid	47.3 ± 3.8[a]	43.6 ± 3.5[a]
	Salicylic acid	21.1 ± 3.0[a]	43.7 ± 7.8[a]
	p-Aminobenzoic acid	37.4 ± 8.8[a]	N.D.
	Propionic acid	17.3 ± 3.4[a]	N.D.
	o-Anisic acid	14.0 ± 4.1[a]	40.6 ± 7.3[a]
Dicarboxylic acids	Glutaric acid	93.9 ± 6.6	80.9 ± 10.3
	Fumaric acid	110 ± 36	82.6 ± 15.6
	Maleic acid	88.2 ± 13.9	88.8 ± 29.9

Note: All carboxylic acids were tested at 10 mM; N.D: not determined.
[a] Significantly different from the control value ($P < 0.05$).

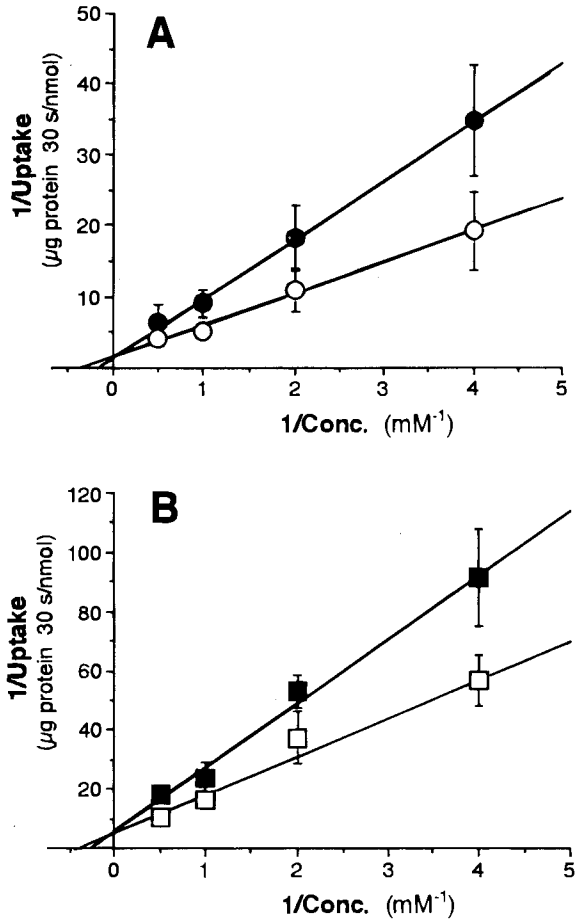

Fig. 3. A: Lineweaver-Burk plots for the uptake of benzoic acid by ROEpi. Uptake was measured in the absence (○) or presence of 10 mM acetic acid (●). B: Lineweaver-Burk plots for the uptake of acetic acid by ROEpi. Uptake was measured in the absence (□) or presence of 10 mM benzoic acid (■). The vertical bar through each point represents the SD of four experiments.

Table 3. Effects of Proteases and Amino Acid-Modifying Agents on [^{14}C]Benzoic Acid Uptake by ROEpi

		Relative uptake (% of control)
Papain	(0.05%)	25 ± 8[a]
Trypsin	(0.001%)	15 ± 3[a]
DTT	(10 mM)	76 ± 10[a]
NEM	(0.5 mM)	64 ± 7[a]

Note: After preincubation with one of these agents, uptake experiments were performed.
[a] Significantly different from the control value ($P < 0.05$).

investigated whether a carrier-mediated transport system exists in oral mucosal epithelial cells. Consequently, we found, for the first time, the existence of a carrier-mediated transport system for monocarboxylic acids in ROEpi. The evidence may be summarized as follows: (a) reduction of the incubation temperature from 37°C to 4°C markedly inhibited the uptake; (b) metabolic inhibitors significantly inhibited the uptake, indicating that the uptake process is energy dependent; (c) monocarboxylic acids significantly inhibited the uptake of benzoic acid or acetic acid; (d) kinetic analysis revealed competitive inhibition by monocarboxylic acid on monocarboxylic acid uptake by ROEpi; (e) the uptake was significantly inhibited when ROEpi were incubated with proteases, or treated with amino acid-modifying agents. These findings strongly suggest the existence of a carrier-mediated monocarboxylic acid transport system in ROEpi.

Caco-2 cells possess a monocarboxylic acid transport system which is pH dependent (6). The activation on benzoic acid uptake by acidic pH can be explained by H^+ cotransport, OH^- exchange system, or a possible change in affinity to the carrier protein depending on the extracellular pH. The lack of an inhibitory effect of DIDS, an inhibitor of anion exchange, suggests that an OH^- exchange system with benzoic acid is not involved. Many types of transporters are Na^+ dependent. However, benzoic acid uptake by ROEpi was not inhibited in the Na^+-free buffer which was prepared by substituting choline chloride for NaCl and NaH_2PO_4 (data not shown). These suggest that the driving force for benzoic acid transport is proton gradient.

Various animal cells take up monocarboxylic acids via transporters present in these cells. To date, the existence of two types of monocarboxylic acid transporter (MCT) in these cells has been reported (13,14). MCT type 1 (MCT1) is expressed in erythrocyte, skeletal muscle cells, and intestinal epithelial cells. MCT type 2 (MCT2) is expressed in hepatocytes. The most striking biochemical difference between MCT1 and MCT2 is the difference in their sensitivity to organomercurial thiol reagents such as *p*-chloromercuribenzoic acid. The inhibitory effect of *p*-chloromercuribenzoic acid on benzoic acid uptake by ROEpi suggests that the transporter in ROEpi may be MCT1, which is also expressed in the small intestine epithelium (data not shown). However, in the present study, we obtained no direct evidence regarding the expression of MCT1, MCT2, or another unknown transporter type in ROEpi.

The physiological role of this carrier-mediated transport of monocarboxylic acid in ROEpi is unclear. However, our present results suggest that drugs which have a monocarboxylic acid residue could be delivered into the systemic circulation from the oral mucosa via its carrier.

This carrier-mediated oral mucosal route may serve the new approach to the absorption of drugs since their degradation by gastrointestinal juices and hepatic first-pass metabolism is avoided.

REFERENCES

1. D. Harris and J. R. Robinson. *J. Pharm. Sci.* **81**:1–10 (1992).
2. M. E. de Vries, H. E. Bodde, J. C. Verhoef, and H. E. Junginger. *Crit. Rev. Ther. Drug Carrier Syst.* **8**:271–303 (1991).
3. I. A. Siegel. *Arch. Oral Biol.* **29**:13–16 (1984).
4. Y. Kurosaki, N. Aya, Y. Okada, T. Nakayama, and T. Kimura. *J. Pharmacobio-Dyn.* **9**:287–296 (1986).
5. Y. Kurosaki, S. Hisaichi, C. Hamada, T. Nakayama, and T. Kimura. *J. Pharmacobio-Dyn.* **10**:180–187 (1987).
6. A. Tsuji, H. Takanaga, I. Tamai, and T. Terasaki. *Pharm. Res.* **11**:30–37 (1994).
7. A. Tsuji, M. T. Simanjuntak, I. Tamai, and T. Terasaki. *J. Pharm. Sci.* **79**:1123–1124 (1990).
8. M. R. Tavakoli-Saberi, and K. L. Audus. *Pharm. Res.* **6**:160–166 (1989).

9. M. R. Tavakoli-Saberi, and K. L. Audus. *Int. J. Pharm.* **56**:135–142 (1989).
10. D. Oda, and E. Watson. *In Vitro Cell. Dev. Biol.* **26**:589–595 (1990).
11. A. Claass, S. Claus, J. Höft, and E. Prange. *Acta Histochem.* **90**:21–26 (1991).
12. D. Z. D'Argenio, and A. Schumitzky. *Comput. Programs Biomed.* **9**:115–134 (1979).
13. C. K. Garcia, J. L. Goldstein, R. K. Pathak, R. G. W. Anderson, and M. S. Brown. *Cell,* **76**:865–873 (1994).
14. C. K. Garcia, M. S. Brown, R. K. Pathak, and, J. L. Goldstein. *J. Biol. Chem.* **270**:1843–1849 (1995).