Possible Role of Anion Exchanger AE2 as the Intestinal Monocarboxylic Acid/Anion Antiporter

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Purpose. The purpose of the present study was to investigate the transport of organic monocarboxylic acids mediated by the anion exchanger AE2, which has been already reported to be present at several tissue cell membranes, including intestinal brush border membrane in rabbit.

Methods. Membrane transport of organic monocarboxylic acids by AE2 was investigated by transient AE2-gene expression in HEK 293 cells and subsequent uptake studies by the cells.

Results. Functional transfection of AE2 was confirmed by the enhanced $^{36}Cl^-$ efflux from the cells. When preloaded with chloride anion, AE2-transfected cells demonstrated a significantly enhanced [^{14}C]benzoic acid transport activity compared with mock-transfected cells. The AE2-mediated uptake was saturable with kinetic parameters of Km = 0.26 ± 0.08 mM and Vmax = 6.14 ± 0.52 nmol/mg protein/3 min, and the uptake of [^{14}C]benzoic acid was pH-dependent with a maximal uptake at pH 6.5. AE2-mediated [^{14}C]benzoic acid uptake was inhibited by Cl^-, HCO_3^-, and DIDS. AE2-transfected cells demonstrated significantly enhanced transport activity for nicotinic acid, propionic acid, butyric acid, and valproic acid as well as benzoic acid compared with mock-transfected cells.

Conclusions. AE2 is functionally involved in the anion antiport for organic monocarboxylic acids as well as inorganic anions and is supposed to play a partial role in the intestinal transport of organic acids.

KEY WORDS: anion exchanger; HEK 293; monocarboxylic acid; intestinal transport; benzoic acid.

INTRODUCTION

Intestinal absorption of organic weak acids has long been ascribed to passive diffusion according to the pH-partition hypothesis. However, we have recently shown that several monocarboxylic acids permeate intestinal brush-border membranes by pH-dependent and carrier-mediated mechanisms, i.e., proton-coupled transport and/or pH-sensitive bicarbonate exchange mechanisms, by using isolated intestinal brush-border membrane vesicles (BBMVs) and intestinal epithelial monolayer of Caco-2 cells (1–8). We have also suggested that MCT1 may contribute as a proton/monocarboxylic acid cotransporter to the intestinal absorption of monocarboxylic acids (8–10). However, molecular identification of the anion antiporter has not been achieved.

The anion exchanger (AE) gene family consists of three members: AE1, AE2, and AE3. These genes encode plasma membrane Cl^-/HCO_3^- exchange proteins which have homologous bipartite structures with a hydrophilic, cytoplasmic amino-terminal domain and a hydrophobic, membrane-spanning carboxyl-terminal domain. The membrane-spanning domains share approximately 65% amino acid identity among family members, whereas the cytoplasmic domains are less closely related. Each isoform exhibits a differential tissue distribution (11), but characterization of their specific functions is still in its early stages.

AE2 was originally isolated from the human erythroleukemic cell line K562 (12) and subsequently from several other tissues, including mouse and human kidney (13,14), rat gastric mucosa (11), mouse choroid plexus (15), and rabbit small intestine (16). Interestingly, AE2 protein was identified by immunoblot analysis in brush-border membrane of rabbit small intestine (16). AE2 and monocarboxylic acid/anion antiporter in intestinal BBMVs are very likely to be structurally related, because both of them are susceptible to inhibition by stilbene disulfonates. Although several groups (17–21) have suggested a possible role of AE1 in transport of organic anions in erythrocyte membranes, little is known about the organic anion transport activity of AE2.

The purpose of the present study was to investigate the activity of AE2-mediated monocarboxylic acid transport by a transient gene expression method using HEK 293 cells. We compared the functional properties of monocarboxylic acid transport mediated by AE2 with the characteristics of the previously proposed monocarboxylic acid/anion-antiport mechanism (5,6,8) in the intestinal brush-border membrane.

MATERIALS AND METHODS

Materials

The HEK 293 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). Mouse AE2 cDNA was a generous gift from Dr. R. Kopito, Stanford University (22). pBK-CMV and pCMVβ-gal were obtained from Stratagene (La Jolla, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY, USA). Penicillin G, streptomycin, and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Valinomycin and [^{14}C]nicotinic acid (2.03 GBq/mmol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). [^{14}C]Benzoic acid (0.56 GBq/mmol), [^{14}C]propionic acid (1.89 GBq/mmol), [^{14}C]butyric acid (0.58 GBq/mmol), [^{14}C]inulin (185 MBq/g), and ^3H_2O (37 MBq/g) were purchased from New England Nuclear (Boston, MA, USA). [^{14}C]Valproic acid (2.04 GBq/mmol) and [^{3}H]aspartic acid (122 GBq/mmol) were purchased from ARC Inc. (St Louis, MO, USA). [^{36}Cl]NaCl (0.11 GBq/g) was purchased from Amersham (Buckinghamshire, England).

Construction of the Expression Vector

Mouse AE2 cDNA (22) (4.1 kbp Nhe I/Hind III restriction fragment) was subcloned into the Nhe I/Hind III site of the

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ABBREVIATIONS: AE, anion exchanger; BBMVs, brush-border membrane vesicles; DIDS, 4,4'-diisothiocyanostilben-2,2'-disulfonic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MES, 2-(*N*-Morpholino)ethanesulfonic acid.

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expression vector pBK-CMV; the orientation of these constructs was confirmed by restriction mapping and sequence analysis. Mock-transfected cells mean the cells transfected with pBK-CMV vector only.

Expression of Anion Exchanger in Cultured Cells

Mouse anion exchanger cDNAs were expressed in HEK 293 cells (23). Cells were grown and transfected in DMEM supplemented with 10% FCS and maintained in a humidified incubator at 37°C and 5% CO_2. HEK 293 cells were cultured for 12–16 h before transfection. Transfection was carried out by adding 20 μg of plasmid DNA per 150-mm dish as a calcium phosphate precipitate (24). After 48 h, the cells were washed once with phosphate-buffered saline (PBS) and used for transport assay after further cultivation for 24–30 h. Transfection efficiency was 32 ± 3% (mean of 3 determinations ± S.E.M.) when assessed by transfecting pCMVβ-gal by the same procedure as above, followed by in situ staining for β-galactosidase activity (25).

$^{36}Cl^-$ Efflux Assay in HEK 293 Cells

Transfected HEK 293 cells were washed in the standard reaction buffer (30 mM NaCl, 16 mM HEPES-KOH, pH 7.4, and 200 mM sucrose) and were resuspended in standard reaction buffer containing 5 μCi of [^{36}Cl]NaCl in the presence of 3 μM valinomycin to depolarize the membrane potential for 20 min at room temperature. The cells were then pelleted by centrifugation at 10,000 rpm for 10 sec and efflux was initiated by resuspending the cells in standard reaction buffer. The reaction was terminated by separating the cells from the medium by a centrifugal filtration technique using a mixture of silicon and liquid paraffin (density 1.03). The resultant pelleted HEK 293 cells were transferred into vials containing scintillation cocktail (Cleasol I; Nacalai Tesque, Kyoto, Japan) and associated radioactivities were determined with a liquid scintillation counter (Aloka, Tokyo, Japan).

Uptake Experiments

Transfected HEK 293 cells were preloaded for 30 min with a loading buffer (90 mM KCl, 46 mM K-gluconate, 1 mM Mg-gluconate, 1 mM Ca-gluconate, 2 mM KH_2PO_4, 10 mM mannitol, 3 μM valinomycin, 0.1% ethanol, and 10 mM HEPES/KOH, pH 7.0). Then, the cells were pelleted and washed once with a transport buffer (130 mM K-gluconate, 1 mM Mg-gluconate, 1 mM Ca-gluconate, 20 mM mannitol, 3 μM valinomycin, 0.1% ethanol, and 10 mM HEPES/KOH, pH 7.0). Transport was initiated by diluting the cells to a concentration of 500,000 cells in 250 μl of transport buffer containing radiolabeled test compounds. At the indicated time, uptake was terminated by centrifugal filtration as described above. Uptake of monocarboxylic acids is shown in terms of the cell-to-medium ratio (C/M ratio) obtained by dividing the uptake amount by the medium concentration, except for the study on the concentration-dependence of transport.

Preparation of Anti-AE2 Polyclonal Antibody and Imunoblotting

Rabbit anti-AE2 polyclonal antibody was raised against a synthetic peptide (CGVDEYNEMPMPV) corresponding to the deduced carboxyl-terminal amino acid sequence of AE2 (13). The peptide was conjugated to keyhole limpet hemocyanin (Sigma), emulsified in an equal volume of Freund's adjuvant (Difco, Detroit, MI, USA), and injected subcutaneously into New Zealand White rabbits. AE2- and mock-transfected HEK 293 cells were harvested by scraping with a rubber policeman and washed in PBS before being lysed in 2% sodium dodecyl sulfate (SDS) solution. Proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membrane (Millipore Ltd., Bedford, MA, USA). Secondary antibodies coupled to alkaline phosphatase were used to localize antibody binding. Immunoblotting was performed by enhanced chemiluminescence (ECL; Amersham).

RESULTS

Expression of Monocarboxylic Acid uptake in *Xenopus laevis* Oocytes injected with Mouse AE2 cRNA

To clarify whether AE2 has the monocarboxylic acid transport activity or not, we first expressed AE2 in a *Xenopus laevis* oocytes heterologous gene expression system. The activities for uptake of [^{14}C]benzoic acid and [^{3}H]acetic acid expressed in AE2 cRNA-injected oocytes were significantly increased compared with those in water-injected oocytes (data not shown). However, since the intrinsic transport activity of oocytes evaluated for water-injected oocytes in the control study was rather high, we decided not to use oocytes to evaluate AE2 activity in the present study. Instead, we employed a transient expression system with HEK 293 cells, which have little Cl^-/HCO_3^- exchange activity (22).

Transient Expression of AE2 in HEK 293 Cells

The AE2 cDNA was inserted into the expression vector pBK-CMV (AE2/pBK-CMV). Anti-AE2 antiserum prepared in our laboratory specifically detected recombinant AE2 as two bands of approximately 165 and 145 kDa in HEK 293 cells transiently transfected with AE2/pBK-CMV vector (Fig.1, lane

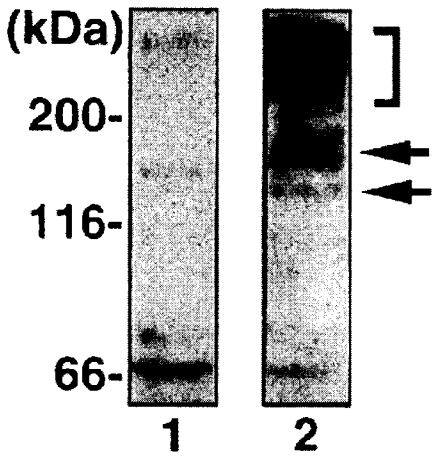

Fig. 1. Western blot analysis of transfected HEK 293 cells. Total proteins (25 μg/lane) from whole mock- (lane 1) and AE2- (lane 2) transfected HEK 293 cells were subjected to 7.5 % SDS-polyacrylamide gel electrophoresis followed by transfer to PVDF membrane. The blot was immunostained with anti-AE2 antibody.

2, arrows). These bands have been previously characterized as the mature and endoplasmic reticulum forms of glycosylated AE2 (26). AE2 protein exhibited a tendency to aggregate to higher order multimers (Fig. 1, bracket), which were previously reported (27). When vector without AE2 was transfected into the cells (mock) as the control, no band corresponding to the two AE2 proteins was detected by Western blot analysis (Fig. 1, lane 1). A previous study using antibody has shown an expression of AE2 at the brush border-membrane in rabbit enterocytes (16). Although, in the present study, the signal of AE2 protein was very slight in murine ileal epithelial cells, AE2 gene specific product was obtained by polymerase chain reaction (data not shown). This may be caused by inadequate sample treatment for Western blotting for the tissue sample, the different portion of the peptide sequence that developed antibody between rabbit (16) and the present study, or relatively lower abundance of antigen in mouse.

To confirm the expression of a functional anion exchange activity of AE2, we measured the efflux of $^{36}Cl^-$ from HEK 293 cells. Efflux of $^{36}Cl^-$ was initiated by incubating the cells in the medium containing 30 mM Cl^-, and the efflux was evaluated from the remaining amount of $^{36}Cl^-$ in the cells. The values of the remaining $^{36}Cl^-$ (% of initial) in AE2- and mock-transfected HEK 293 cells were 58.5 ± 2.6% and 105.2 ± 2.6% (mean of 3 determinations ± S.E.M.), respectively, at 1 min, showing an enhanced efflux in AE2-transfected cells.

Time Course of the Uptake of Benzoic Acid by HEK 293 Cells

Figure 2 shows the time courses of [^{14}C]benzoic acid uptake by HEK 293 cells transfected with AE2 or expression vector alone. AE2-transfected cells, when preloaded with 90 mM Cl^-, exhibited significantly increased and time-dependent uptake, while no time-dependent uptake was observed in the mock-transfected cells. The uptake by AE2-transfected cells linearly increased for 3 min, so all subsequent initial uptake measurements were performed at 3 min after preloading with 90 mM Cl^-. Cell volumes of AE2- and mock-transfected HEK 293 cells, evaluated by calculating the difference of apparent uptakes of ^3H_2O and [^{14}C]inulin, were 8.01 ± 1.10 μl/mg and 11.3 ± 2.13 μl/mg (mean of 6 determinations ± S. E. M.), respectively.

Inhibition of Benzoic Acid Uptake by DIDS and by Inorganic Anions

Specific inhibition of AE2 activity was studied to confirm that benzoic acid uptake by HEK 293 cells is mediated by AE2. As is clearly shown in Table 1, the enhanced uptake of [^{14}C]benzoic acid was significantly decreased in the presence of DIDS. Furthermore, it was remarkably decreased in the presence of chloride or bicarbonate ion.

Concentration Dependence of Benzoic Acid Transport

The concentration dependency of the initial uptake of benzoic acid was examined. To evaluate AE2-derived uptake, the result was expressed after subtraction of the uptake by mock-transfected cells from that by AE2-transfected cells (Fig. 3). From Eadie-Hofstee analysis (shown in Fig. 3, inset), a single saturable process was apparently involved in AE2-transfected HEK 293 cells. Nonlinear least-squares analysis of the result yielded an apparent Km of 0.26 ± 0.08 mM and Vmax of 6.14 ± 0.52 nmol/mg protein/3 min.

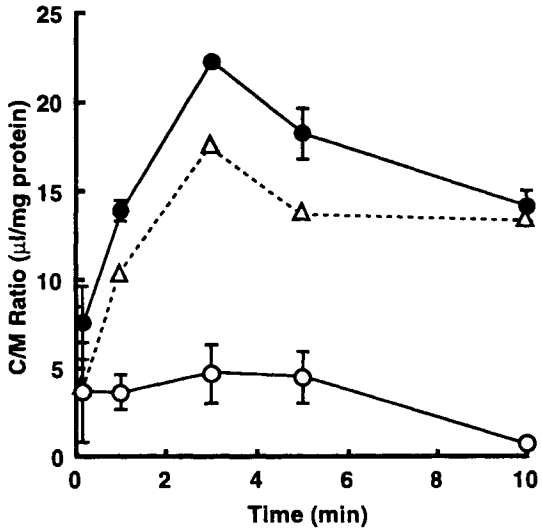

Fig. 2. Time course of [^{14}C]benzoic acid uptake in AE2-transfected HEK 293 cells. AE2- (closed circles) and mock- (open circles) transfected HEK 293 cells were preloaded with 10 mM HEPES-KOH buffer (pH 7.0) containing 90 mM KCl at 37°C. The uptake of [^{14}C]benzoic acid (67 μM) was measured at 37°C in 10 mM HEPES-KOH buffer (pH 7.0) containing 130 mM K-gluconate. Each solution contained an appropriate concentration of mannitol to be isotonic. All experiments were performed in the medium containing 3 μM valinomycin and 0.1% ethanol. The uptake shown by open triangles is the AE2-dependent uptake obtained as the difference between the uptakes in AE2- and mock-transfected HEK 293 cells. Each point represents the mean ± S.E.M. of 3 to 4 experiments.

Effect of Extracellular pH on Benzoic Acid Uptake

The pH dependence of [^{14}C]benzoic acid transport mediated by AE2- or mock-transfected HEK 293 cells is shown in Fig. 4. The uptake of [^{14}C]benzoic acid increased with decreasing pH in both cases. AE2-mediated uptake, obtained by sub-

Table 1. Effects of Inorganic Anions and Anion Exchange Inhibitor on the Uptake of [^{14}C]Benzoic Acid in Mouse AE2-Transfected HEK 293 Cells

Inhibitor	Concentration (mM)	Uptake (μl/mg/3min)	
		Mock-transfected	AE2-transfected
K-Gluconate	130	5.42 ± 2.84	30.88 ± 1.37[a]
Chloride	10	2.74 ± 2.41	2.18 ± 2.52
Bicarbonate	10	0.00 ± 1.47	1.18 ± 0.86
DIDS	0.2	3.68 ± 2.80	5.27 ± 2.43

Note: Uptake measurements of AE2- and mock-transfected HEK 293 cells were conducted as described in the legend to Fig. 2. Transport of [^{14}C]benzoic acid (67 μM) was measured at 37°C in 10 mM chloride-free HEPES-KOH buffer (pH 7.0) containing each inhibitor. Each value represents the mean ± S.E.M. of 4 determinations.

[a] Significantly different from the uptake by mock-transfected cells by Student's t-test ($p < 0.05$).

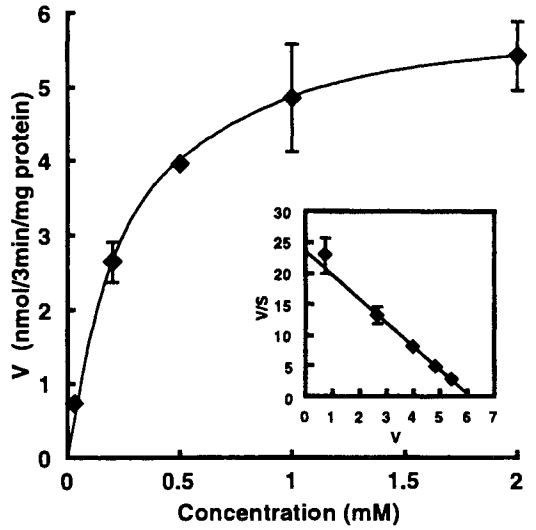

Fig. 3. Concentration dependence of benzoic acid uptake in HEK 293 cells. The uptake of [^{14}C]benzoic acid in AE2- and mock-transfected HEK 293 cells preloaded with 90 mM chloride ion was measured for 3 min at 37°C in chloride-free 10 mM HEPES-KOH buffer (pH 7.0). Each solution contained an appropriate concentration of mannitol to be isotonic. The data were obtained by subtraction of the uptake by mock-transfected cells from that by AE2-transfected cells. Inset: Eadie-Hofstee plot for the saturable process of concentration-dependent benzoic acid uptake. Each point represents the mean ± S.E.M. of 3 to 6 experiments.

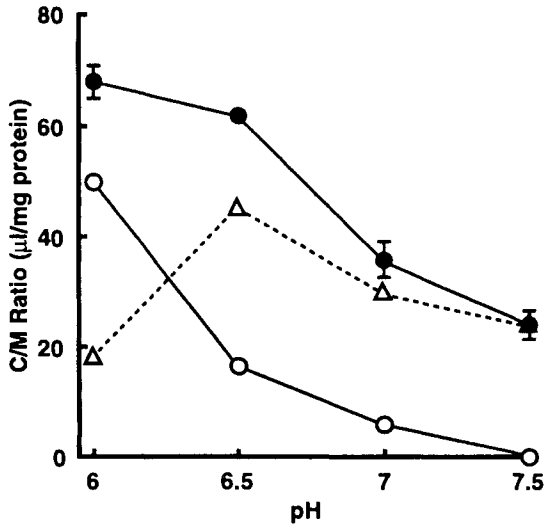

Fig. 4. Extracellular pH dependence of benzoic acid uptake in HEK 293 cells. The uptake of [^{14}C]benzoic acid in AE2- (closed circles) and mock- (open circles) transfected HEK 293 cells preloaded with 90 mM chloride ion was measured for 3 min at 37°C in chloride-free 10 mM MES-KOH (pH 6.0) or HEPES-KOH buffer (pH 6.5, 7.0, 7.5) containing 130 mM K-gluconate. Each solution contained an appropriate concentration of mannitol to be isotonic. The uptake shown by open triangles is the AE2-dependent uptake obtained as the difference between the uptakes in AE2- and mock-transfected HEK 293 cells. Each point represents the mean ± S.E.M. of 3 experiments.

traction of uptake by mock-transfected cells from that by AE2-transfected cells, was optimum at pH 6.5.

Transport of Various Monocarboxylic Acids in HEK 293 Cells

Substrate specificity of AE2 was assessed by measuring the transport of several monocarboxylic acids. Uptakes of [^{14}C]benzoic acid, [^{14}C]nicotinic acid, [^{14}C]propionic acid, [^{14}C]butyric acid and [^{14}C]valproic acid by AE2-transfected HEK 293 cells were significantly increased compared with mock-transfected cells (Table 2), whereas the uptake of an acidic amino acid, [^{3}H]aspartic acid, was not increased by AE2-transfection.

DISCUSSION

The present study was designed to examine whether the anion exchanger AE2 transports organic monocarboxylic acids by exchanging them with bicarbonate and/or chloride ions, since firstly AE2 has been shown to be present at the brush-border membrane by immunoblot analysis (16), secondary AE2 is sensitive to DIDS and has a transport function to exchange anions such as Cl^-, HCO_3^-, NO_3^-, and SO_4^{2-} (22,26,28,29), and lastly AE1, a member of the same family as AE2, has been suggested to transport organic anions (17-21).

A heterologous gene expression system, *Xenopus laevis* oocytes, expressed transport activity after AE2-cRNA injection. However, since the oocytes also showed a similar intrinsic activity, we used HEK 293 cells to evaluate AE2 function. We confirmed the functional expression of AE2 in HEK 293 cells by immunoblot analysis and measurement of Cl^-/Cl^- exchange activity, and we demonstrated that AE2-transfected HEK 293 cells exhibit significantly enhanced benzoic acid transport activity compared with mock-transfected cells. The transport activity was DIDS-sensitive. The enhanced [^{14}C]benzoic acid uptake in AE2-transfected HEK 293 cells cannot be ascribed to cell volume change after transfection, since no significant difference in cell volume

Table 2. Uptakes of Monocarboxylic Acids in AE2-Transfected HEK 293 Cells

Monocarboxylic acid	Uptake (μl/mg/3min)	
	Mock-transfected	AE2-transfected
Benzoic acid	3.44 ± 0.99	23.72 ± 5.22[a]
Nicotinic acid	0.66 ± 0.52	5.83 ± 1.03[a]
Propionic acid	4.74 ± 5.38	32.67 ± 3.45[a]
Butyric acid	11.03 ± 0.37	24.62 ± 1.35[a]
Valproic acid	10.15 ± 1.72	26.25 ± 3.86[a]
Aspartic acid	11.08 ± 1.57	8.69 ± 1.32

Note: Uptakes of [^{14}C]benzoic acid (67 μM), [^{14}C]nicotinic acid (26 μM), [^{14}C]propionic acid (20 μM), [^{14}C]butyric acid (63 μM), [^{14}C]valproic acid (18 μM) and [^{3}H]aspartic acid (30 nM) were measured by incubating AE2- and mock-transfected HEK 293 cells in chloride-free 10 mM HEPES-KOH buffer (pH 7.0). Each value represents the mean ± S.E.M. of 3 to 4 determinations. All experiments were performed under identical conditions as described in the legend to Fig. 2.

[a] Significantly different from the uptake by mock-transfected cells by Student's t-test ($p < 0.05$).

between AE2- (8.01 μl/mg protein) and mock-transfected (11.3 μl/mg protein) HEK 293 cells was seen. The $^{36}Cl^-$ efflux from AE2-transfected HEK 293 cells was enhanced in medium containing benzoic acid compared with medium containing K-gluconic acid (data not shown). Although there is the possibility that an efficient pH-change in cells caused by AE2 may affect the uptake of benzoic acid, these results demonstrate that the enhanced uptake of [^{14}C]benzoic acid in AE2-transfected HEK 293 cells can be ascribed to the transport by AE2 protein.

In this study, AE2-transfected HEK 293 cells significantly enhanced transport of several monocarboxylic acids, such as benzoic acid, nicotinic acid, propionic acid, butyric acid, and valproic acid, as compared with mock-transfected cells. However, an acidic amino acid, aspartic acid, was not transported by AE2. We have previously shown that uptakes of most of these compounds were enhanced by imposing an outward bicarbonate gradient in rabbit intestinal BBMVs (6). Accordingly, the intestinal monocarboxylic acid/anion antiporter appears to have a similar substrate specificity to AE2.

In rabbit small intestine, Cl^-/HCO_3^- exchange activity has been shown to be present only on the brush-border surface of crypt and villus cells (30). In addition, at the brush-border membrane predominant AE2 signal was demonstrated by immunoblot analysis of rabbit ileum (16). Saturable kinetics and the relatively acidic optimal pH of 6.5, which is close to small intestinal microclimate pH, observed in AE2-mediated benzoic acid transport are consistent with those for transport of other monocarboxylic acids in the intestine (6). At present, since benzoic acid transport across the intestinal brush-border membrane has not been characterized in detail, a precise comparison of the transport characteristics at the brush-border membrane with those of AE2, cannot be made.

This study suggested that the benzoic acid transport activity via AE2 protein is affected by chloride and bicarbonate ions. Considering the physiological concentration of these anions, there is the possibility that inorganic anions in intestinal lumens may not provide an optimal condition for AE2-mediated monocarboxylic acid absorption. Since AE2 is supposed to be functionally bi-directional, it may facilitate secretion of monocarboxylic acids from enterocytes to lumen.

AE2 is widely distributed among various tissues and cultured cell lines (11-16) in addition to small intestine. Accordingly, it is possible that AE2 proteins may contribute to the transport of various monocarboxylic acids and play an important role in the transport of organic acidic drugs and nutrients in various tissues where AE2 is present.

In conclusion, in the present study, it was demonstrated that AE2 protein functions as a pH-dependent organic monocarboxylic acid transporter.

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