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Changes in the permeation rate of organic anions through the intestinal brush-border membrane with membrane surface potential

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Abstract

The effects of membrane surface potential on the uptake of anionic compounds by rat intestinal brush-border membrane vesicles were investigated. The uptake amount of all tested anionic compounds (ceftibuten, cefixime, benzylpenicillin, s-1006 and rentiapril) in the neutral medium (pH 7.5) was lower than that in the acidic medium (pH 5.5). Changes in surface potential of brush-border membrane vesicles were monitored using a fluorescence dye, 8-anilino-1-naphthalenesulfonate (ANS), and the results suggested an increase of a negative charge on the membrane surface proportional to the increase of the pH of medium. A good correlation was observed between the initial uptake rate of all tested anionic compounds and relative membrane surface potential monitored by ANS. Moreover, the uptake of cefixime by artificial liposome made from PC containing various amount of DPPS was measured. The uptake value of cefixime was decreased in proportion to an increase of DPPS content. These results suggest that the permeation of anionic compounds across intestinal brush-border membrane is dependent on surface potential originate in the surface negative charge.

Key words: Organic anion; Brush-border membrane; Surface potential; Permeation

1. Introduction

The permeation rates of organic acids through the small intestinal epithelium has been explained by pH-partition hypothesis in that the permeability of the substances through the cell membrane in the unionized form depends on the pH of the solutions and the pK_a of the substances [1]. However, Hogben et al. [2] and Hogerle and Winne [3] observed a deviation of the intestinal drug absorption from the pH-partition hypothesis, i.e., the pH-absorption curves were less steep than expected and were shifted to higher pH values for organic acids. They pointed out the effects of unstirred layer and the microclimate pH [4.5], as a reason for this deviation. It is impossible, however, to explain the reason for the pH-dependent absorption of organic acids which possess low pK_a values and is completely ionized in the physiological pH region, approx. 5–7.5, by the effect of unstirred layer and the microclimate pH. On the other hand, Simanjuntak et al. [6] and Tsuji et al. [7] proposed the carrier system for monocarboxylic acid transport across the brush-border membrane. The details of the transport characteristics of these organic acids still remain unclear. Recently, we reported that some of the organic cations taken up into the brush-border membrane vesicles were driven by membrane potential differences (inside-negative K^+- and H^+-diffusion potential) [8–10]. Moreover, stimulation by inside-negative K^+-diffusion potential was observed in the uptake of amphiphilic organic cations [11,12]. Therefore, it is reasonable that membrane potential also affects the permeation of organic anions similar to organic cations, although they possess different charges (positive or negative). However, there are few reports that investigate the variations of permeation rate from viewpoint of the membrane potential. In this study, the effect of membrane potential (ionic

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; PC, phosphatidylcholine; DPPS, dipalmitoylphosphatidylserine.

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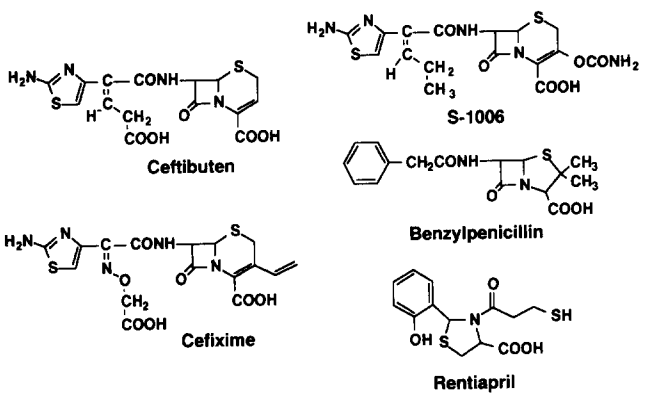

Fig. 1. Structures of tested ionic compounds.

diffusion potential and surface potential) on the uptake of anionic compounds (Fig. 1) across intestinal brush-border membrane was investigated, in order to elucidate the permeation mechanism common to organic anions.

2. Materials and methods

2.1. Chemicals

Ceftibuten, S-1006 (Shionogi, Osaka, Japan), cefixime (Fujisawa Pharmaceutical, Osaka, Japan) and Rentiapril (Santen, Osaka, Japan) were kindly donated. Benzyl[^{14}C]penicillin potassium (spec. act. 2.0 GBq/mmol) was purchased from Amersham (Bucks, UK). Valinomycin, egg-yolk phosphatidylcholine (PC) and DL-α-phosphatidylserine, dipalmitoyl (DPPS) were purchased from Sigma (St. Louis, MO, USA). 8-Anilino-1-naphthalenesulfonate magnesium (ANS) was obtained from Nakalai Tesque (Kyoto, Japan). All other chemicals were of the highest grade available and used without further purification.

2.2. Preparation of intestinal brush-border membrane vesicles

Brush-border membrane vesicles were isolated from rat whole intestine by $CaCl_2$ precipitation [13] as described previously [14]. Membrane vesicles were suspended in the buffer used for the transport studies. The composition of the buffer is given in the figure legends.

2.3. Preparation of liposomes (large unilamellar vesicles)

Liposomes were prepared by the reversed phase evaporation technique [15]. The lipid mixture (80 mg total lipid) in chloroform were added to a 50 ml round-bottommed flask, and solvent was removed by a rotary evaporator. The lipids were redissolved in 6 ml of diethyl ether. When the lipids were indissoluble, an adequate amount of chloroform was added. 1.5 ml of a buffer (100 mM D-mannitol, 100 mM KCl, 20 mM Hepes-Tris (pH 7.5)) was added to the organic solution and the mixture was sonicated in a bath-type sonicator (UT-204, Sharp, Osaka, Japan), under nitrogen for 5 min. The mixture was then placed on a rotary evaporator and the organic solvent was removed under vacuum. Following the addition of 3 ml of above-mentioned buffer, the suspension was evaporated to remove the traces of organic solvent.

2.4. Uptake experiments

The uptake of substrates was measured by a rapid filtration technique as described previously [16]. When the brush-border membrane vesicles were used, the reaction was initiated by addition of 100 μl of a buffer containing the substrate to 20 μl of membrane vesicles suspension (10–15 mg protein/ml) at 25°C. At a predetermined time, the reaction was stopped by diluting the reaction mixture with 5 ml of ice-cold buffer (150 mM NaCl, 20 mM Hepes-Tris buffer (pH 7.5)). The tube contents were immediately filtered through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter) which was washed once with 8 ml of the same ice-cold buffer. In case of the liposomes, the reaction was performed by addition of 100 μl of substrate solution to 50 μl of liposomes suspension at 37°C. After 5 min, the mixture was diluted with 2 ml of ice-cold buffer and immediately filtrated. The filter trapped the vesicles was washed once with 3 ml of the ice-cold buffer. The substrate trapped on the filter was extracted with 300 μl of distilled water and was measured by HPLC or liquid scintillation counting.

2.5. Analytical method

The concentrations of ceftibuten, s-1006 and cefixime were determined by HPLC (Hitachi L-6000) equipped with an L-4000 UV detector (Hitachi, Tokyo, Japan) with detection at 262 nm for ceftibuten and s-1006, and 280 nm for cefixime. Rentiapril was determined by HPLC at an applied voltage of + 850 mV vs. Ag/AgCl using ECD-100 electrochemical detector (Eicom, Kyoto, Japan). Separation was achieved on a reversed phase column (ODS, Hitachi 3053, $d_p = 5$ μm, 4 mm i.d., 250 mm) using a mobile phase consisting of methanol/0.05 M citrate buffer (pH 2.5) (1:9; ceftibuten, 15:85; s-1006 and cefixime, 11:89; rentiapril). The limit of detection was 2 pmol for ceftibuten and cefixime, 4 pmol for s-1006 and rentiapril. Protein concentrations were determined by the method of Lowry et al. [17] with bovine serum albumin as the standard. Phospholipid was determined by the method of Bartlett [18].

2.6. Measurement of surface potential change of membrane vesicles

Changes in the surface potential were monitored by measuring the changes in the fluorescence intensity of ANS, which has been widely used to measure surface charge density and surface potential of the membranes [19–21]. The measurements were carried out in a spectrofluorometer (650-60, Hitachi, Tokyo, Japan) with an excitation wavelength of 385 nm and emission wavelength of 480 nm. The temperature was maintained at 25°C. To 1 ml of membrane vesicle suspension, 1 ml of dye solution was added. Final concentrations of vesicles and ANS were 0.1 mg protein/ml and 30 μM for brush-border membrane vesicles and 0.75 μmol phospholipid/ml and 50 μM for liposomes, respectively. Following which the fluorescence intensity in each various pH of medium was measured. Corrections for background fluorescence and light scattering were made with blanks containing brush-border membranes alone and dye alone. The composition of the buffer is given in the figure legend.

3. Results

3.1. Effect of ionic diffusion potential on the uptake of anionic compounds by rat intestinal brush-border membrane vesicles

Both Yoshikawa et al. [22] and the authors [23] have reported that the initial uptake of ceftibuten was stimulated remarkably and an overshoot phenomenon was observed in the presence of an inward directed H^+ gradient. However, it is unknown whether the H^+-diffusion potential contribute to the uptake of ceftibuten or not. Fig. 2 shows the initial uptake of ceftibuten (30 s) in the presence of an inwardly directed H^+ gradient using voltage clamped vesicles. Under this condition, H^+-diffusion potential is immediately compensated by K^+ movement as described previously [10]. The initial uptake of ceftibuten in the presence of inwardly directed H^+ gradient was not decreased, even though the H^+-diffusion potential was dissipated. The effect of a valinomycin-induced inside-positive K^+-diffusion potential on the uptake of dianionic (ceftibuten and cefixime) and monoanionic (s-1006) compounds was also examined (Figs. 3, 4), and results suggest that the uptakes of these anionic compounds were not affected by ionic diffusion potential.

3.2. Effect of medium pH on the uptake of anionic compounds by rat intestinal brush-border membrane vesicles

The effect of medium pH on the uptake of ceftibuten, cefixime and benzylpenicillin is shown in Fig. 5. Common to these anionic compounds, the uptake amount in the acidic medium (pH 5.5) was higher than that in the neutral medium (pH 7.5). Fig. 6 shows the effect of medium osmolarity on the uptake of ceftibuten and benzylpenicillin in the acidic and neutral medium. The uptake in the acidic medium was decreased with an increase in the medium osmolarity. These results suggest that differences of uptake values in the acidic and neutral medium are due to the differences of the permeation rates through the brush-border membrane. Fig. 7 shows the time-course of the uptake of s-1006 and rentiapril in the presence or absence of an H^+ gradient. In this case, although

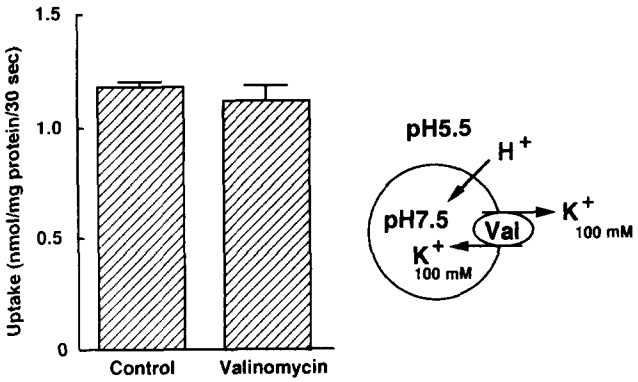

Fig. 2. Effect of dissipation of H^+-diffusion potential on the uptake of ceftibuten in the presence of inwardly directed H^+ gradient by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM D-mannitol, 100 mM KCl and 20 mM Hepes-Tris buffer (pH 7.5) in the presence or absence of valinomycin (7 μg/mg protein). The vesicles (20 μl) were incubated with 100 μl of 20 mM Mes-Tris buffer (pH 5.5), containing 1.2 mM ceftibuten 100 mM D-mannitol and 100 mM KCl. Each point represents the mean ±S.E. of four measurements.

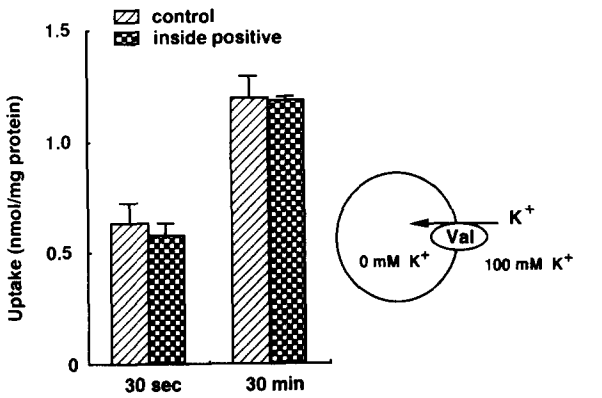

Fig. 3. Effect of K^+-diffusion potential (inside-positive) on the uptake of ceftibuten by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM D-mannitol, 100 mM Na-gluconate and 20 mM Mes-Tris buffer (pH 7.5) in the presence of valinomycin (7 μg/mg protein). The vesicles (20 μl) were incubated with 100 μl of 20 mM Mes-Tris buffer (pH 5.5), containing 1.2 mM ceftibuten, 100 mM D-mannitol and either 100 mM Na-gluconate (control) or K-gluconate (inside-positive). Each point represents the mean ±S.E. of three or four measurements.

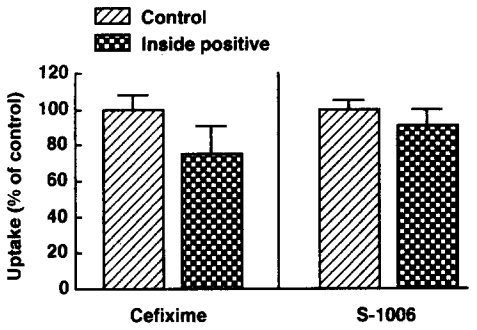

Fig. 4. Effect of K^+-diffusion potential (inside-positive) on the uptake of cefixime and s-1006 by rat intestinal brush-border membrane vesicles. The experimental conditions are as given in Fig. 2. Each point represents the mean ±S.E. of three or four measurements.

overshoot phenomenon was not observed, there was difference of the uptake rate similar to other compounds (ceftibuten, cefixime and benzylpenicillin).

3.3. Relationship between the uptake of anionic compounds and membrane surface potential

Changes in the fluorescence intensity of ANS as a function of membrane surface potential in the various pH of medium were measured. As shown in Fig. 8, the

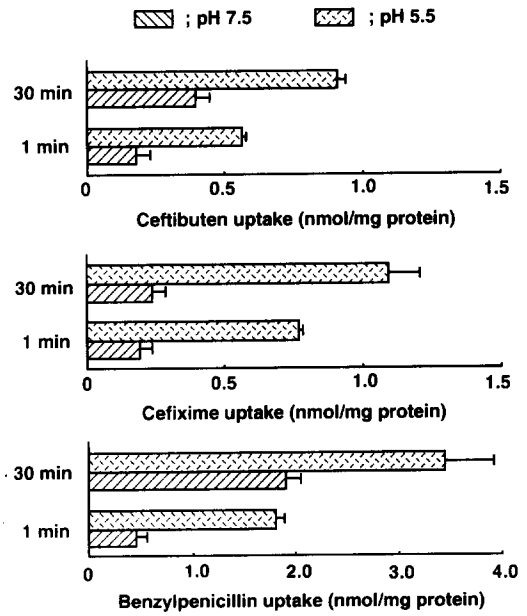

Fig. 5. Effect of medium pH on the uptake of ceftibuten, cefixime and benzylpenicillin by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM D-mannitol, 100 mM KCl and either 20 mM Hepes-Tris buffer (pH 7.5) or 20 mM Mes-Tris buffer (pH 5.5). The vesicles (20 μl) were incubated with 100 μl of the same buffer containing each organic anion (1.2 mM for ceftibuten and cefixime, 2.4 mM for benzylpenicillin). Each point represents the mean ±S.E. of 3–6 measurements.

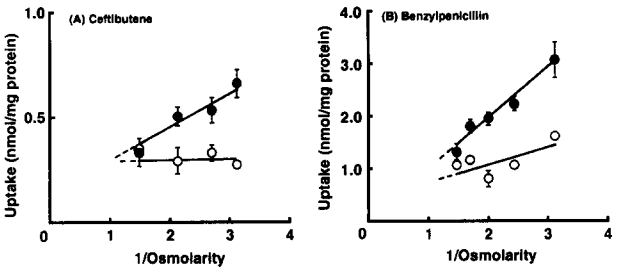

Fig. 6. Effect of medium osmolarity on the uptake of ceftibuten (A) and benzylpenicillin (B) by rat intestinal brush-border membrane vesicles. Membrane vesicles were preincubated in 100 mM D-mannitol, 100 mM KCl and either 20 mM Hepes-Tris buffer (pH 7.5) or 20 mM Mes-Tris buffer (pH 5.5). The vesicles (20 μl) were incubated with 100 μl of the same buffer containing organic anion (1.2 mM) and various concentrations of D-cellobiose (○, pH 7.5; ●, pH 5.5). Each point represents the mean ±S.E. of three or four measurements.

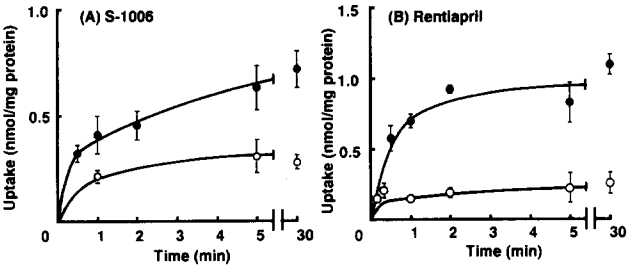

Fig. 7. Time-course of uptake of s-1006 (A) and rentiapril (B) by rat intestinal brush-border membrane vesicles in the presence (●) or absence (○) of H^+ gradient. Membrane vesicles were preincubated in 100 mM D-mannitol, 100 mM KCl and 20 mM Hepes-Tris buffer (pH 7.5). The vesicles (20 μl) were incubated with 100 μl of either 20 mM Hepes-Tris buffer (pH 7.5) or 20 mM Mes-Tris buffer (pH 5.5) containing 100 mM D-mannitol 100 mM KCl and either 1.2 mM s-1006 or 1.2 mM rentiapril. Each point represents the mean ±S.E. of 3–6 measurements.

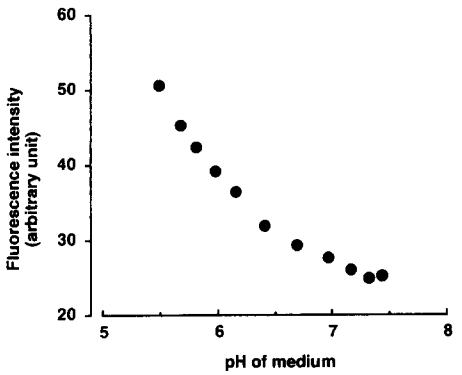

Fig. 8. Changes in the fluorescence intensity of ANS as a function of surface potential in rat intestinal brush-border membrane vesicles. The vesicles were suspended in 100 mM D-mannitol, 100 mM KCl and either 20 mM Hepes-Tris buffer ($7.0 \leq pH \leq 7.5$) or 20 mM Mes-Tris buffer ($5.5 \leq pH < 7.0$). To 1 ml of vesicle suspension, 1 ml of same buffer containing 60 μM ANS was added and fluorescence intensity was measured.

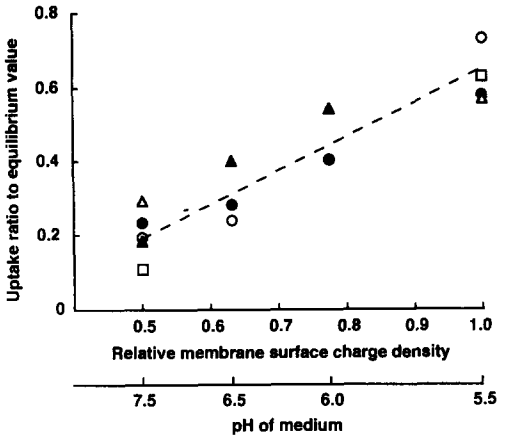

Fig. 9. The relationship between the initial uptake of anionic compounds by the intestinal brush-border membrane vesicles and the membrane surface potential. The membrane surface potential is expressed as the ratio to the degree on pH 5.5. ●, ceftibuten; ○, cefixime; ▲, benzylpenicillin; △, s-1006; □, rentiapril.

flourescene intensity of ANS was decreased with the increase of the medium pH. This result suggest the increase of the negative surface charge on the intestinal brush-border membrane by a rise in the pH of medium. The relation between the initial uptake rate and the relative negativity of the membrane surface potential monitored by ANS at the various pH is shown in Fig. 9. The vertical axis indicates the ratio of the initial uptake value (1 min) at the various pH levels to the uptake value for 30 min at a standard of pH 5.5. A good correlation was observed and it was common to the anionic compounds tested. Furthermore, the uptakes of cefixime by liposomes containing various amount of DPPS were measured. As shown in Fig. 10, the uptake of cefixime was also correlated with fluorescence intensity of ANS.

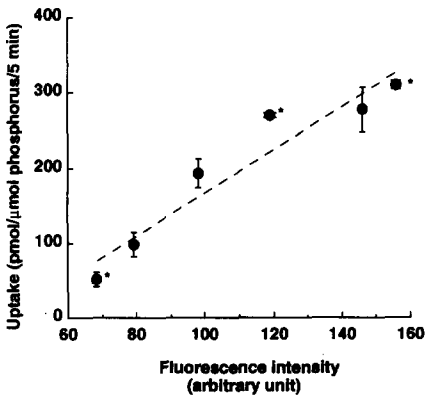

Fig. 10. The relationship between the uptake of cefixime by the liposomes and the membrane surface potential. Each point represents the mean ±S.E. of three measurements or mean ±range of two measurements (*).

4. Discussion

The effects of membrane surface potential on the uptake of organic anions by rat intestinal brush-border membrane vesicles were investigated. Recently, we observed the H^+- and K^+-diffusion potential dependent uptake of organic cations [8–10]. In this study, inside-positive K^+-diffusion potential did not stimulate the uptake of anionic compounds (ceftibuten, cefixime and s-1006) which are highly ionized at the physiological pH (Figs. 3, 4). Moreover, the initial uptake of ceftibuten in the presence of inwardly directed H^+ gradient did not decrease even though the voltage-clamped vesicles were used (Fig. 2). These results suggest that the ionic diffusion potential does not take part directly in the uptake of these organic anions. It was obviously different from the case of organic cations. On the other hand, common to the tested anionic compounds, a pH-dependent uptake was observed (Figs. 5, 7). The permeation rate through the brush-border membrane was also dependent on the medium pH (Fig. 7). These results, inefficacy of the ionic diffusion potential and the pH-dependent uptake behaviors, suggest the possibility that the change of the membrane properties i.e., membrane surface charge, occurs and participates in the permeation of these organic anions, because these anionic compounds were nearly all ionized in the pH range examined. Therefore, changes in the membrane surface potential were monitored by ANS, a fluorescent dye and a marker for the membrane surface potential. The fluorescence intensity of ANS lowered with the increase of the medium pH, suggesting that the increase of negative charge on the membrane surface in the neutral pH. Common to the tested anionic compounds, a good correlation was observed between the initial uptake rate and relative membrane surface potential monitored by ANS. Moreover, the uptake of cefixime by liposomes, which does not include any protein, was decreased with increase of DPPS content (Fig. 10). These results suggest that the surface potential plays an important role in the pH-dependent permeation of organic anions through the intestinal brush-border membrane. An electrostatic repelling force between the organic anions and negative charges on the membrane surface might hinder the permeation of the substances in the neutral pH.

In conclusion, it was suggested that the permeation of some organic anions is dependent on the membrane surface potential, but not on the ionic diffusion potential.

5. References

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