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Effect of membrane surface potential on the uptake of anionic compounds by liposomes

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Abstract

The effect of membrane surface potential on the uptake of several anionic compounds by liposomes (large unilamellar vesicles), which contain various amounts of dipalmitoylphosphatidylserine (DPPS), was investigated. The uptake amount of four tested anionic compounds (cefixime, benzyloxyindoleacetic acid (BOIAA), ceftibuten and S-1006) decreased with an increase in the DPPS content of liposomes, and was correlated with the membrane surface potential monitored using a fluorescent dye, 8-anilino-1-naphthalene sulfonate (ANS). Moreover, for all of the tested anionic compounds, a good correlation was observed between the ratio of the uptake value (5 min) by each of the liposomes comprising various amounts of DPPS to the uptake value by liposomes containing 10% DPPS and a relative membrane surface potential monitored by ANS. On the other hand, the uptake of zwitterionic compounds (enoxacin, cephradine and benzyloxytryptophan (BOTP)) was independent of DPPS content. These results suggest that the uptake of tested anionic compounds by large unilamellar lipid vesicles is dependent on the membrane surface potential which originates in the surface negative charge.

Key words: Organic anion; Membrane; Permeation; Surface potential; Liposome

1. Introduction

The relative permeation of organic acids through the epithelial cell membrane, which is important as the first step of intestinal drug absorption, has usually been explained in terms of the pH-partition hypothesis; that weak electrolytes permeate through the lipid membrane in the un-ionized form [1]. It is useful for the estimation of intestinal absorption to measure the organic solvent/water partition coefficient using the solvents with a varying polarity [2–4]. However, as there have been some highly anomalous compounds, it is necessary to take other factor into consideration to understand the mechanisms of intestinal absorption. Hogben et al. [5] and Hogerle and Winne [6] pointed out that the deviation between the pH-absorption curve and the pH-partition curve is ascribed to the effects of the unstirred layer and microclimate-pH [7,8]. Davis et al. [9] reported that ion-pair formation contributes to the good intestinal absorption of an acidic drug, proxicromil. On the other hand, since the epithelial cell membrane has a net negative surface charge, it is quite probable that the electrostatic interaction between the membrane and anionic drugs affects their permeation. However, there are few studies concerning membrane permeation from a viewpoint of electrostatic drug-membrane interaction for anionic compounds.

Recently, Alcorn et al. [10] reported that the partitioning of ionizable drugs with intestinal brush-border membrane is much higher than would be expected from the organic solvent distribution due to 'surfactant-like' ionic interactions with the membrane and its surface charges. The authors reported that the uptake amount of several mono- and di-anionic compounds by intestinal brush-border membrane vesicles decreased with the increase of the pH of the medium, even though these anionic compounds nearly all ionized at

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Abbreviations: Hepes, *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; ANS, 8-anilino-1-naphthalenesulfonate; DPPS, DL-α-phosphatidylserine dipalmitoyl; BOIAA, 5-benzyloxyindole-3-acetic acid; BOTP, 5-benzyloxy-DL-tryptophan.

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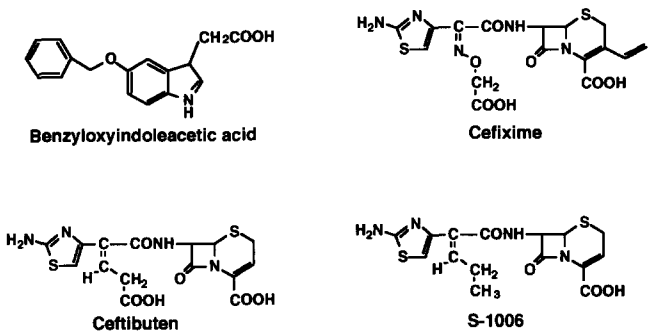

Fig. 1. Structures of tested anionic compounds.

tested pH [11,12]. Moreover, the alteration in the uptake of the anionic compounds depended on the change of the membrane surface potential monitored by ANS [12]. However, strictly speaking, when brush-border membrane vesicles are used the effect of the change of the medium pH to the proportion of un-ionized form and contribution of carrier-mediated transport [13,14] on the uptake cannot be disregarded. In this study further investigation by uptake experiment using large unilamellar vesicles containing various amount of DPPS was performed to clarify the relation between membrane permeation of anionic compounds (Fig. 1) and membrane surface potential. Under these conditions, there is no protein and alteration of the pH of the medium is not required.

2. Materials and methods

2.1. Chemicals

Cefixime (Fujisawa Pharmaceutical, Osaka, Japan), Ceftibuten, S-1006 (Shionogi, Osaka, Japan), Enoxacin (Dainippon Pharmaceutical, Osaka, Japan) and Cephradine (Sankyo, Tokyo, Japan) were kindly donated. 5-Benzyloxyindole-3-acetic acid (BOIAA), 5-Benzyloxy-DL-tryptophan (BOTP), Egg yolk phosphatidylcholine 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.

2.2. Preparation of liposomes (large unilamellar vesicles)

The liposomes were prepared by a reversed phase evaporation technique [15]. The lipid mixture (80 mg total lipid) in chloroform was added to a 50 ml round-bottom 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 vortex mixing and the addition of 3 ml of the above-mentioned buffer, the suspension was evaporated to remove traces of the organic solvent.

2.3. Uptake experiments

The uptake of substrates was measured by a rapid filtration technique as described previously [12,16]. The reaction was initiated by the addition of 100 μl of substrate solution to 50 μl of a liposome suspension at 37°C. At a predetermined time, the reaction was stopped by diluting the reaction mixture with 2 ml of ice-cold buffer (150 mM NaCl, 20 mM Hepes-Tris (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 3 ml of the same ice-cold buffer. The substrate trapped on the filter was extracted and measured by HPLC. The solution used for the extraction of substrates was as follows: distilled water 0.3 ml; cefixime, ceftibuten, S-1006 and cephradine, 20 mM Hepes-Tris buffer (pH 7.5) containing 150 mM NaCl 2 ml; BOIAA, 1 ml; BOTP, 0.2 M acetic acid 0.4 ml; enoxacin.

2.4. Analytical method

The concentrations of cefixime, ceftibuten, S-1006, enoxacin and cephradine were determined by an HPLC (L-6000, Hitachi, Tokyo Japan) equipped with an L-4000 UV detector (Hitachi) with detection at 262 nm for ceftibuten, S-1006 and cephradine, 280 nm for cefixime, and 265 nm for enoxacin. BOIAA and BOTP were determined by HPLC at an excitation wavelength of 285 nm and an emission wavelength of 350 nm using an F-1050 fluorescence spectrophotometer (Hitachi). Separation was achieved on a reversed phase column (ODS, Hitachi 3053, 5 μm, 4 mm i.d., 250 mm) using mobile phase consisting of acetnitril: 0.05 M citrate buffer, pH 2.5 (1:4; cefixime, ceftibuten and s-1006, 1:3; BOIAA), acetnitril: 0.05 M phosphate buffer, pH 6.0 (1:4; BOTP), methanol: 0.05 M phosphate buffer, pH 6.0 (1:4; cephradine) and methanol: 0.05 M potassium dihydrogenphosphate containing 2% acetic acid (3:7; enoxacin). Phospholipids were determined following the method of Bartlett [17].

2.5. Measurement of membrane surface potential change of liposomes

Changes in the membrane surface potential of liposomes were monitored by measuring the changes in the fluorescence intensity of ANS, which has been widely used to measure the membrane surface potential of the bio- and artificial membranes [18–20], as described previously [12]. The measurements were carried out in a spectrofluorometer (650-60, Hitachi) with an excitation wavelength of 385 nm and emission wavelength of 480 nm. The temperature was maintained at 37°C. To 1 ml of the liposome suspension, 1 ml of dye solution was added and the fluorescence intensity was measured. The final concentrations of liposomes and fluorescent dye were 0.75 μmol pospholipid/ml, and 50 μM, respectively. Corrections for background fluorescence and light scattering were made with blanks containing liposomes alone and dye alone.

Fluorescence intensity, f, is defined as

$$f = f_a - (f_d + f_m) \quad (1)$$

where f_a, f_d and f_m are fluorescence intensity of liposome-ANS suspension, ANS solution alone and liposome suspension alone, respectively. Relative membrane surface potential at a standard of 10% DPPS liposome, ψ_{rel}, was calculated by the following equation

$$\psi_{rel} = f_l / f_s \quad (2)$$

where f_l and f_s stand for fluorescence intensity calculated by Eq. (1) using liposomes containing various amount of DPPS and standard liposome (10% DPPS), respectively.

3. Results

3.1. Time-course of the uptake of anionic compounds by liposomes

Fig. 2 shows the time-course of the uptake of anionic compounds, BOIAA (Fig. 2(A)) and cefixime

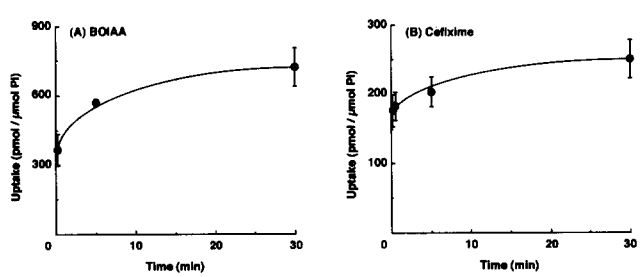

Fig. 2. Time-course of the uptake of BOIAA (A) and cefixime (B) by liposomes containing 20% DPPS. The liposomes (50 μl) were incubated with 100 μl of 20 mM Hepes-Tris buffer (pH 7.5), containing 100 mM D-mannitol, 100 mM KCl and either 0.6 mM BOIAA or 1.2 mM cefixime at 37°C. Each point represents the mean ± S.E. of three or four measurements.

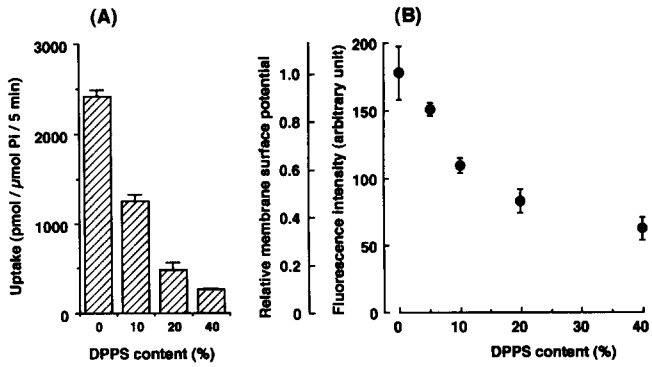

Fig. 3. (A) The uptake of BOIAA by liposomes containing various amounts of DPPS. The liposomes (50 μl) were incubated with 100 μl of 20 mM Hepes-Tris buffer (pH 7.5), containing 100 mM D-mannitol, 100 mM KCl and 0.6 mM BOIAA at 37°C. Each value represents the mean with S.E. of three measurements. (B) Changes in the fluorescence intensity of ANS as a function of the surface potential in liposomes containing various amounts of DPPS. Experimental procedure is described in Materials and methods. Each point represents the mean ± S.E. of three measurements.

(Fig. 2(B)), by liposomes containing 20% DPPS. Although there was a difference in the uptake amount between BOIAA and cefixime, which might be due to the difference of lipophilicity of these compounds, both anionic compounds were certainly taken up by liposomes. Moreover, it was confirmed that these liposomes are closed by measuring the valinomycin-induced potassium diffusion potential, which changes linearly with the logarithmic concentration of KCl, using a fluorescent dye, diS-C_3(5), as a probe (not shown). These results suggest that the liposomes prepared for this study are useful for the evaluation of the uptake properties of these drugs.

3.2. Effect of membrane surface potential on the uptake of BOIAA

To clarify the relationship between the uptake of anionic compounds and membrane surface potential, the uptake of BOIAA by liposomes which contained various amounts of DPPS was measured. As shown in Fig. 3(A), the uptake amount of BOIAA decreased with an increase in DPPS content in the liposomes. Fig. 3(B) shows changes in the fluorescence intensity of ANS, which reflects the membrane surface potential of liposomes as a function of the DPPS content. The fluorescence intensity of ANS decreased with an increase in DPPS content, suggesting an increase of membrane surface potential. The relationship between the relative membrane surface potential at a standard of liposomes containing 10% DPPS and the uptake of BOIAA is shown in Fig. 4. A good correlation was observed and these results suggest that the uptake of BOIAA by liposomes is dependent on the membrane surface potential.

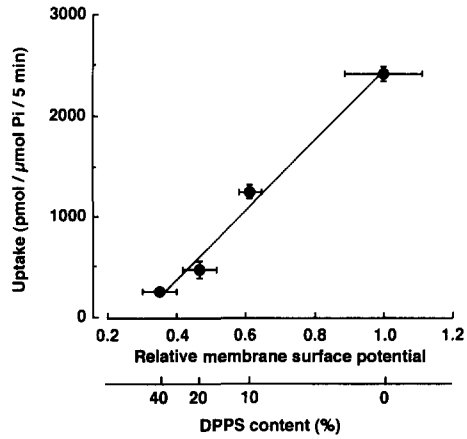

Fig. 4. The relationship between the uptake of BOIAA by liposomes and relative membrane surface potential. The values of uptake and fluorescence intensity from Fig. 3 are used.

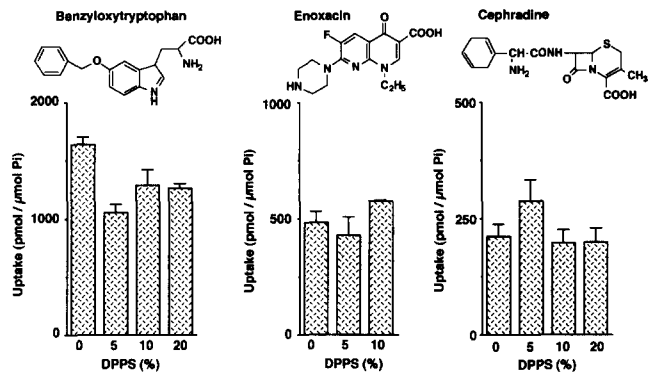

Fig. 6. Effect of DPPS content on the uptake of zwitterionic compounds. The liposomes (50 μl) were incubated with 100 μl of 20 mM Hepes-Tris buffer (pH 7.5), containing 100 mM D-mannitol, 100 mM KCl and one of zwitterionic compounds (0.375 mM BOTP, 1.2 mM enoxacin, 1.2 mM cephradine) at 37°C. Each value represents the mean with S.E. of three measurements.

3.3. Common occurrence of membrane surface potential-dependent uptake of anionic compounds

To clarify whether membrane surface potential dependent uptake is observed in other anionic compounds, the uptake of cefixime, ceftibuten and S-1006 by liposomes was measured. Fig. 5 shows the relationship between the membrane surface potential and the uptake of tested anionic compounds. The horizontal and the vertical axes indicate the relative membrane surface potential, and the ratio of the uptake value (5 min) by liposomes with various DPPS contents to the uptake value at a standard of liposomes containing 10% DPPS, respectively. For all of the tested anionic compounds, a good correlation was observed between the relative membrane surface potential and the uptake ratio, and they all appeared to be more or less constant.

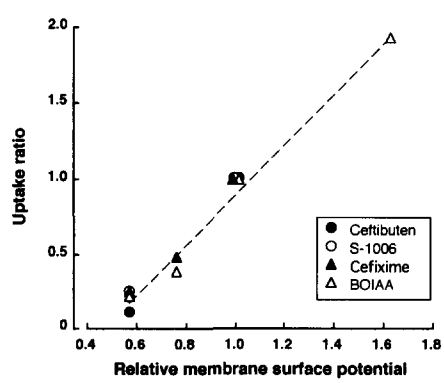

Fig. 5. The relationship between the uptake of anionic compounds and relative membrane surface potential. The uptake and the membrane surface potential are expressed as the ratio to the degree of liposomes containing 10% DPPS. The liposomes (50 μl) were incubated with 100 μl of 20 mM Hepes-Tris buffer (pH 7.5), containing 100 mM D-mannitol, 100 mM KCl and one of anionic compounds (1.2 mM; ceftibuten, s-1006 and cefixime, 0.6 mM; BOIAA) at 37°C. Each point represents the mean of 3–6 measurements.

3.4. Effect of membrane surface potential on the uptake of zwitterionic compounds

Fig. 6 shows the effect of DPPS content on the uptake of zwitterionic compounds (BOTP, enoxacin and cephradine), by liposomes. In contrast to anionic compounds, the uptake of these zwitterionic compounds was not affected by the DPPS content, suggesting that the uptake of zwitterionic compounds are independent of membrane surface potential.

4. Discussion

Recently, we have investigated the effect of ion diffusion potential and membrane surface potential on the uptake of anionic compounds by intestinal brush-border membrane vesicles. It was observed that the uptake of anionic compounds is dependent on the membrane surface potential, but is not affected by ion diffusion potential, which stimulates the electrophoretic permeation of organic cations [12,21–24]. However, because the composition of biomembrane is very complex and the membrane surface potential might therefore be strongly influenced by the change of medium pH, it is difficult to investigate only the effect of membrane surface potential. Therefore, we used liposomes (large unilamellar vesicles) to clarify the effect of membrane surface potential on the uptake of anionic compounds. With this artificial membrane, it is possible to change the membrane surface potential by the addition of various amounts of DPPS without altering the medium pH.

In the case of small unilamellar vesicles, asymmetry of charged lipids in mixed phospholipid systems with phosphatidylcholine has been observed. It is generally agreed that this asymmetry is related to curvature effects arising from the small radius of the vesicles [25,26]. In order to get rid of this asymmetry, Eastman et al. used LUV for the investigation of the transbilayer movement of phosphatidic acid [26]. Since we use LUV in this study, the effect of asymmetry of the phospholipid bilayer on the uptake and fluorescence measurement should be negligible.

Fluorescence intensity of ANS is affected by both membrane surface potential and ion diffusion potential [18,27–30]. However, Schafer and Rowohl-Quisthoudt, and Aiuchi et al. have reported that changes in the ANS fluorescence in isolated mitochondria are mainly attributed to changes in the membrane surface potential [18,27]. In the present study, because there is no ion diffusion potential (i.e., interior and exterior of the liposomes were equilibrated by the same buffer), changes in the ANS fluorescence should reflect changes in the membrane surface potential of LUV.

As shown in Fig. 2, the time-course of uptake of these anionic compounds exhibit an apparently biphasic manner. Alcorn et al. have reported that the partition of drugs to intestinal brush-border membrane vesicles equilibrate relatively quickly [31]. Recently the authors have observed that the initial fast uptake of chlorpromazine and polyamines is caused by binding to the lipid membrane [32,33]. Therefore, the total uptake of these anionic compounds might be estimated as the sum of the initial partition-dominant phase and following permeation into the vesicle interior. However, a method for separating the partition to the membrane and the permeation into the vesicle interior has not been established, so the uptake in this study includes both partition and permeation. Although the separation of these two factors should be investigated hereafter, the difference of the uptake values between the tested anionic compounds should be due to their different lipophilicity. Actually, the log P (P: n-octanol/buffer (the medium for uptake experiment) partition coefficient) of BOIAA and cefixime are 2.41 $\pm$ 0.55 and -4.45 ± 0.04 (mean $\pm$ S.D., $n = 3$), respectively. In the present study, therefore, in order to eliminate this effect, we compared the ratio of uptake values for various amounts of DPPS to the uptake value at a standard 10% DPPS. As shown in Fig. 5, for all of the four tested compounds, the uptake ratio decreased with an increase of membrane surface potential. This result is consistent with our previous observations using intestinal brush-border membrane vesicles [12]. On the other hand, the uptake of zwitterionic compounds by liposomes was not affected by the DPPS content (Fig. 6). It is well known that the intestinal brush-border membrane has a highly negative surface charge which originates from acidic lipids, sialic acid, and so on [34–36]. We observed that order of uptake rates for several structural analogues by intestinal brush-border membrane vesicles is: cations > zwitterions > anions [24,37 and unpublished data]. These previous results, and the results of this study suggest that there is an electrostatic interaction between ionic compounds and the membrane. Especially with respect to anionic compounds, an electrostatic repelling force takes an important role in permeation through the epithelial cell membrane.

In conclusion, it is suggested that the uptake of some organic anions by liposomes is dependent on the membrane surface potential. This mechanism may actually be an important factor in permeation through the biomembrane for anionic compounds.

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