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Differential sensitivity of organic anion transporters in rat renal brush-border membrane to diethyl pyrocarbonate

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Abstract

The effect of various chemical modifiers on *p*-aminohippurate (PAH) uptake by a potential-sensitive system and by an anion exchanger was studied in rat renal brush-border membrane vesicles. Among various chemical modifiers, diethyl pyrocarbonate (DEPC) selectively inhibited potential-sensitive PAH uptake but not the uptake by the anion exchanger. The inhibitory effect of DEPC on potential-sensitive PAH uptake was not due to the facilitated dissipation of membrane potential, which was evidenced by the studies with a potential-sensitive fluorescence dye diS-C_3(5). The potential-sensitive PAH uptake was inhibited by DEPC in a concentration-dependent manner, and kinetic analysis showed that the decreased uptake of PAH in DEPC-treated vesicles was due to the decrease of V_{max}. The inhibition of the PAH uptake was protected by the presence of organic anions during the DEPC treatment. These findings indicate that PAH transport by the potential-sensitive system and by the anion exchanger is mediated by structurally distinct transporters. Amino acid residue(s) modified by DEPC, most likely a histidine residue, should play an important role in the potential-sensitive transport of PAH in rat renal brush-border membrane.

Key words: Renal transport; Organic anion; *p*-Aminohippurate; Potential-sensitive transport; Anion exchanger; Chemical modification

1. Introduction

Organic anion transport systems of the renal proximal tubule play an important role in the elimination of a wide variety of anionic compounds including endogenous metabolites, drugs, and xenobiotics [1]. Secretion of organic anions such as *p*-aminohippurate (PAH) involves the uptake of the anions from blood into the cells across basolateral membrane and exit into the lumen across brush-border membrane [2]. Studies with isolated membrane vesicles have revealed many aspects of the transport mechanisms of PAH across renal epithelial cells [3].

In rats and dogs, the transport of PAH in renal brush-border membrane had been reported to occur by an anion exchanger which transports various organic and inorganic anions such as urate, lactate, OH^-, Cl^- as substrates [4,5]. On the other hand, in rabbits and pigs, the transport of organic anions such as PAH and urate in renal brush-border membrane reportedly occurs by a specific pathway which is sensitive to a membrane potential, but not by the anion exchanger [6,7]. However, as discussed elsewhere [8], the anion exchanger can not necessarily explain the secretory flux of PAH, rather the system is energetically situated to drive organic anion reabsorption. We recently found that not only the anion exchanger but also the potential-sensitive transport system is involved in PAH transport in rat renal brush-border membrane [9]. These two transport systems were different in their sensitivity to anion transport inhibitors and membrane potential, and we speculated that the potential-sensitive system has an important role for the secretion of PAH across brush-border membrane in rats because the luminal

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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fluid compartment has more electrical positive potential than the intracellular compartment. However, in order to substantiate the involvement of two different transport systems for PAH, it is necessary to show that these transport systems are mediated by structurally distinct transporters or by different parts of one transporter (these two possibilities were not distinguished further in this study).

Chemical modification techniques for amino acid residues have been successfully employed for characterizing a number of different transport systems [10–14]. Therefore in the present study, we examined the effect of various chemical modifiers on PAH uptake by the potential-sensitive transport system and by the anion exchanger in rat renal brush-border membrane vesicles. Among various chemical modifiers tested, diethyl pyrocarbonate (DEPC), a histidine specific reagent, selectively inhibited PAH uptake by the potential-sensitive transport system but did not affect the uptake by the anion exchanger. Thus, our results indicate that in rat renal brush-border membrane, PAH is transported by two distinct transporters, a potential-sensitive transporter and an anion exchanger.

2. Materials and methods

2.1. Preparation of brush-border membrane vesicles

Brush-border membrane vesicles were isolated from the renal cortex of male Wistar albino rats (200–230 g) by the Mg/EGTA precipitation method as described previously [15]. The isolated membrane vesicles were suspended in a buffer comprising 100 mM mannitol and 10 mM Hepes (pH 7.5), and stored at −80°C until use (usually less than 1 week).

2.2. Transport studies

The uptake of [^{14}C]PAH by brush-border membrane vesicles was measured by a rapid filtration technique [16]. For the measurement of potential-sensitive uptake of PAH, membrane vesicles were suspended in 300 mM mannitol and 10 mM Hepes (pH 7.5). After preincubation for 10 min at 25°C, the reaction was rapidly initiated by the addition of a buffer (180 μl) containing 100 mM K gluconate, valinomycin (7–13 μg/mg protein), and 0.055 mM [^{14}C]PAH (final 0.05 mM) to 20 μl of the membrane suspension. For the measurement of PAH uptake by the anion exchanger, membrane vesicles were suspended and preincubated for 70 min at 25°C in a buffer comprising 100 mM mannitol, 100 mM K gluconate, 10 mM Hepes (pH 7.5) and 10 mM unlabeled PAH in order to load the vesicles with PAH. Then the reaction was initiated by the addition of a buffer (180 μl) containing 100 mM K gluconate and 0.055 mM [^{14}C]PAH to 20 μl of the membrane suspension. In both cases, incubation was stopped at the stated times by diluting the reaction mixture with 1 ml of ice-cold stop solution comprising 150 mM KCl and 20 mM Hepes-Tris (pH 7.5). The contents of the tube were immediately poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter) and washed once with 5 ml of ice-cold stop solution. In separate experiments, nonspecific adsorption was estimated by the addition of labeled substrate mixture to 1 ml of ice-cold stop solution containing 20 μl of membrane vesicles. This value was subtracted from the uptake data for background correction.

2.3. Chemical modification

The treatment of brush-border membrane vesicles with *N,N'*-dicyclohexylcarbodiimide (DCCD), phenylglyoxal (PGO), and DEPC was carried out at 25°C for 10 min (DEPC) or for 30 min (DCCD, PGO) as described previously [14], and after washing the vesicles, transport studies were performed. Stock solutions of DCCD and DEPC were prepared fresh daily in ethanol and control experiments were performed with an equivalent amount of ethanol.

2.4. Measurement of fluorescence

A fluorescent dye, 3,3'-dipropylthiadicarbocyanine iodide (diS-C_3(5)), was employed for the evaluation of membrane potential across brush-border membrane vesicles. Briefly, 50–100 μl of brush-border membrane vesicles (3 mg of protein per ml) were added into 3 ml of buffer containing 1.5 μM diS-C_3(5). The fluorescence change was continuously recorded (excitation, 622 nm; emission, 670 nm) while the mixture was stirred at 25°C with a Shimadzu spectrofluorophotometer RF-5000 (Kyoto, Japan).

2.5. Radioactivity and protein determinations

The radioactivity was determined by liquid scintillation counting with an external standard to correct for quench. Protein was determined, after precipitation with ice-cold 10% (w/v) trichloroacetic acid, by the method of Lowry et al. [17] with bovine serum albumin as a standard.

2.6. Materials

p-[*glycyl*-1-^{14}C]Aminohippurate (PAH, 1.5–1.6 GBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA, USA). DEPC and PGO were obtained from Sigma (St. Louis, MO, USA). diS-C_3(5) was obtained from Aldrich (Milwaukee, WI, USA). Hepes, Tris and DCCD were obtained from Nacalai

Tesque (Kyoto, Japan). All other chemicals used were of the highest purity available.

3. Results and discussion

First, we examined the effect of various chemical modifiers on PAH uptake by renal brush-border membrane vesicles. The furosemide-sensitive uptake of PAH was measured in the presence of either inside-positive membrane potential (Fig. 1A) or unlabeled PAH inside the vesicles (Fig. 1B), to evaluate the transport activity by a potential-sensitive system and by an anion exchanger ([^{14}C]PAH/PAH exchange), respectively [9]. In the presence of furosemide, PAH uptake was almost the same under all the conditions, indicating that the treatment of brush-border membrane vesicles with these chemical modifiers does not affect the nonspecific flux of PAH. As shown in Fig. 1, DCCD, a carboxyl group specific reagent, did not affect PAH uptake under both uptake conditions. Treatment with PGO, an arginine residue specific reagent, resulted in a slight but significant inhibition of PAH uptake both by the potential-sensitive system and by the anion exchanger. In contrast, the potential-sensitive PAH uptake but not the anion exchange was markedly inhibited by the treatment of the membrane vesicles with DEPC, a histidine specific reagent. Under the same treatment condition, the uptake of tetraethylammonium, an organic cation, was significantly decreased by all of these chemical modifiers as described previously [14].

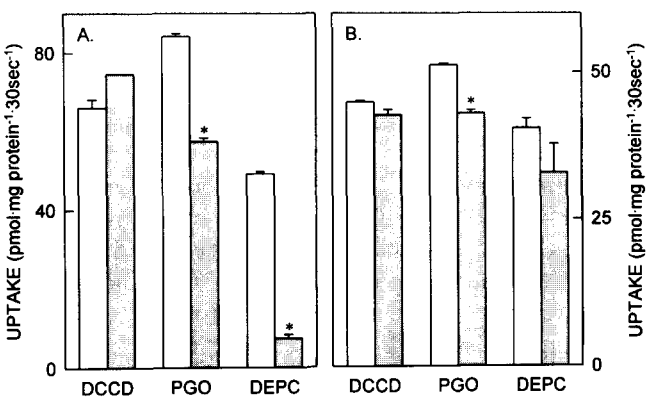

Fig. 1. Effect of various chemical modifiers on PAH uptake by brush-border membrane vesicles. Brush-border membrane vesicles were treated in the absence (open column) or presence (dotted column) of either 0.05 mM DCCD, 1 mM PGO, or 1 mM DEPC. (A) For the measurement of potential-sensitive PAH uptake, membrane vesicles (20 μl), suspended in 300 mM mannitol, 10 mM Hepes (pH 7.5), were incubated for 30 s with the substrate mixture (180 μl) comprising 100 mM mannitol, 100 mM K gluconate, 10 mM Hepes (pH 7.5), valinomycin and 0.055 mM [^{14}C]PAH. (B) For the measurement of PAH uptake by the anion exchanger, membrane vesicles (20 μl), suspended in 100 mM mannitol, 100 mM K gluconate, 10 mM Hepes (pH 7.5) and 10 mM unlabeled PAH, were incubated for 30 s with the substrate mixture (180 μl) comprising 100 mM mannitol, 100 mM K gluconate, 10 mM Hepes (pH 7.5) and 0.055 mM [^{14}C]PAH. In A and B, each uptake was measured either in the absence or presence of 1 mM furosemide, and the data are shown as furosemide-sensitive uptake of PAH. In the presence of furosemide, PAH uptake was essentially the same under all the conditions, and were 14.1±0.6 (A) and 14.3±0.9 (B) pmol (mg protein)$^{-1}$(30 s)$^{-1}$ (n = 6, mean±S.E.). Each column represents the mean±S.E. of three determinations from a typical experiment. * $P < 0.05$, significant difference from control using Student's t-test.

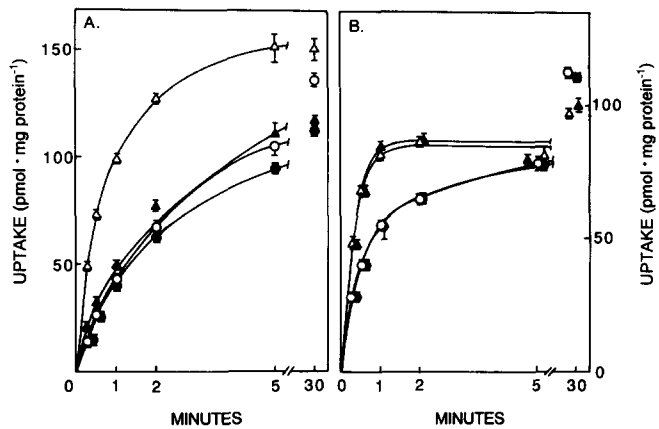

Fig. 2. Effect of DEPC treatment on PAH uptake by brush-border membrane vesicles. (A) Potential-sensitive uptake of PAH was measured as described in Fig. 1. Membrane vesicles were pretreated in the absence (○,△) or presence (●,▲) of 1 mM DEPC and the time-course of [^{14}C]PAH uptake was measured in the absence (○,●) or presence (△,▲) of valinomycin. Each point represents the mean ±S.E. of six determinations from two experiments. (B) PAH uptake by the anion exchanger was measured as described in Fig. 1. Membrane vesicles were pretreated in the absence (○,△) or presence (●,▲) of 1 mM DEPC. The vesicles were then preloaded with (△,▲) or without (○,●) 10 mM PAH and the time-course of [^{14}C]PAH uptake was measured. Each point represents the mean±S.E. of six determinations from two experiments.

Based on the above results, DEPC may be useful to differentiate two transport systems for PAH in rat renal brush-border membrane. In the following studies, we therefore examined the effect of DEPC treatment on PAH uptake further. Fig. 2 shows the effect of DEPC treatment on the time-course of PAH uptake by brush-border membrane vesicles. In Fig. 2A, PAH uptake was measured either in the presence or absence of inside-positive membrane potential, which was created by applying an inward K^+ gradient and valinomycin. PAH uptake was stimulated in the presence of valinomycin compared with that in the absence of valinomycin, confirming the involvement of the potential-sensitive transport system. In brush-border membrane vesicles pretreated with DEPC, the uptake in the presence of valinomycin was markedly decreased compared with that in control vesicles, and the uptake was similar with that in the absence of valinomycin in control vesicles. In the absence of valinomycin, no effect of DEPC was observed. Thus, DEPC treatment

inhibited the potential-stimulated component of PAH uptake but not the uptake in the absence of membrane potential. We have previously shown that even in the absence of inside-positive membrane potential (similar condition with that in the absence of valinomycin in control vesicles in Fig. 2A), PAH uptake was inhibited by furosemide [9]. Therefore, PAH uptake in the absence of valinomycin in Fig. 2A may occur by a specifically mediated pathway other than the potential-sensitive system, most likely by the anion exchanger ([^{14}C]PAH/OH^- exchange) because the exchanger was insensitive to DEPC as described below. The insensitivity to DEPC of the uptake in the absence of valinomycin further indicates that the potential-sensitive transport system for PAH may be inactive when the membrane potential was not applied. Fig. 2B shows the effect of DEPC on PAH uptake by the anion exchanger. PAH uptake was measured either in the presence or absence of unlabeled PAH preloaded in the vesicles. The uptake of [^{14}C]PAH was stimulated by preloading the vesicles with 10 mM of unlabeled PAH (trans-stimulation by [^{14}C]PAH/PAH exchange), confirming the involvement of the anion exchanger. In contrast to the potential-sensitive PAH uptake, however, DEPC treatment did not affect PAH uptake whether or not the vesicles were preloaded with unlabeled PAH, suggesting that the anion exchanger is insensitive to DEPC treatment.

There are a few reports on the effect of chemical modification on PAH uptake by renal brush-border membrane. Sokol et al. [18,19] showed that sulfhydryl, arginyl, and histidyl groups are essential for organic anion exchange in canine renal brush-border membrane. The inhibitory effect of PGO treatment on PAH uptake observed in our study may be compatible with their results. On the other hand, the effect of DEPC is different in two studies, because we found no effect of DEPC treatment on PAH uptake by the anion exchanger. The reason for this discrepancy is not clear, but it may suggest that the anion exchanger in rat and canine renal brush-border membrane may be structurally different.

The uptake of PAH by brush-border membrane vesicles was linearly related to the magnitude of inside-positive membrane potential [9]. Therefore, it is necessary to test whether the inhibition of potential-sensitive PAH uptake by DEPC treatment is due to its effect on membrane potential. To examine this possibility, a potential-sensitive fluorescence dye, diS-C_3(5), was employed. diS-C_3(5) has been used successfully to characterize the nature of various transport systems such as Na^+-dependent D-glucose transport [20,21]. We first examined the effect of glucose on the fluorescence of diS-C_3(5) to confirm the applicability of the dye in our experimental settings. After equilibration of the membrane vesicle suspension with Na^+ and diS-C_3(5), either D-or L-glucose was added. The fluorescence of the dye was increased by the addition of D-glucose but not L-glucose, which represents a stereospecific, electrogenic cotransport of D-glucose with Na^+ as described previously [20]. As can be expected, the fluorescence increase of the dye could not be observed when D-glucose was added to the membrane suspension in the absence of Na^+ (data not shown). Using this fluorescence dye, we therefore examined the effect of DEPC treatment on the magnitude of an inside-positive membrane potential created by K^+ gradient and valinomycin. The membrane vesicle suspension was equilibrated with the dye and valinomycin, and then K gluconate was added. As shown in Fig. 3, the magnitude of fluorescence increase induced by K^+ was similar in control and DEPC-treated membrane vesicles. These results indicate that the inhibitory effect of DEPC treatment on the potential-sensitive PAH transport was not due to the consequence of impaired creation and/or facilitated dissipation of the membrane potential, but rather DEPC should modify the essential amino acid residues in the transporter and inhibit PAH transport.

Fig. 4 shows the effect of DEPC concentration on PAH uptake by brush-border membrane vesicles. The vesicles were treated with various concentrations of DEPC, and PAH uptake for 30 s and 90 min (equilibrium uptake) was measured in the presence of inside-positive membrane potential. The equilibrium up-

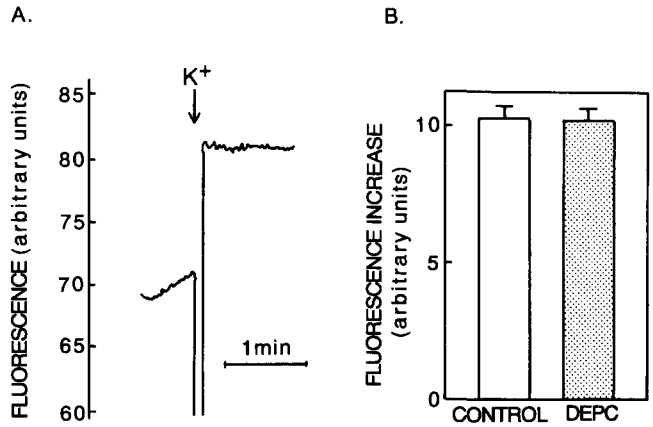

Fig. 3. Effect of DEPC treatment of brush-border membrane vesicles on the fluorescence change of diS-C_3(5) induced by K^+ gradient and valinomycin. (A) Brush-border membrane vesicles (3 mg/ml, 50 μl), suspended in 1 mM K gluconate, 99 mM Na gluconate and 10 mM Hepes (pH 7.5), were added in the reaction mixture (3 ml) comprising 1 mM K gluconate, 99 mM Na gluconate, 10 mM Hepes (pH 7.5), valinomycin (6 μg/ml) and 1.5 μM diS-C_3(5). At the time indicated by the arrow, K gluconate (50 μl, final concentration was 33 mM) was added in the mixture, and the change in fluorescence was measured. (B) The fluorescence change of diS-C_3(5) induced by K^+ gradient and valinomycin was measured in control or DEPC-treated (DEPC) brush-border membrane vesicles. Each column represents the mean ± S.E. of three determinations from a typical experiment.

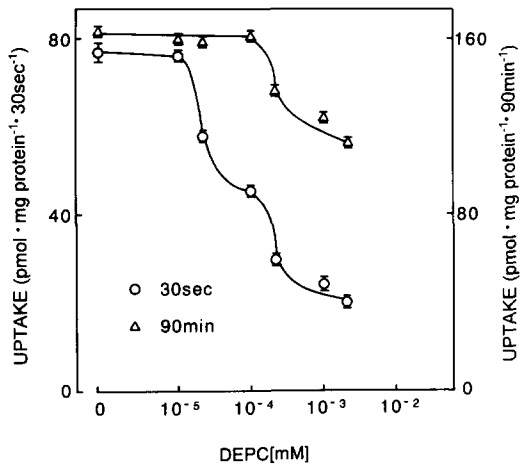

Fig. 4. Effect of DEPC concentration on the potential-sensitive uptake of PAH by brush-border membrane vesicles. Brush-border membrane vesicles were pretreated with various concentrations of DEPC, and the potential-sensitive uptake of PAH for 30 s (○) and for 90 min (△) was measured. Each point represents the mean ± S.E. of six determinations from two experiments.

take was achieved at around 30 min and then was not changed appreciably for up to 90 min (data not shown). The PAH uptake for 30 s was inhibited by DEPC treatment at the concentrations higher than 0.05 mM in a concentration-dependent manner. When DEPC concentrations higher than 0.5 mM were used, the equilibrium uptake of PAH was also decreased, though the magnitude of decrease was less than that of 30 s uptake. Therefore, at least DEPC concentrations up to 0.1 mM, DEPC inhibited PAH uptake without affecting the intravesicular volume and/or the integrity of membrane vesicles.

The concentration dependence of potential-sensitive PAH uptake was examined in control and DEPC-treated brush-border membrane vesicles. In these experiments, PAH uptake for 5 s was measured in the presence or absence of valinomycin, and the latter was subtracted from the former to exclude the uptake in the absence of membrane potential, which may be mediated by the system other than the potential-sensitive transport system as described above. As shown in Fig. 5, potential-sensitive component of PAH uptake was saturable in both membrane vesicles, and the Eadie-Hofstee plot of the data showed a linear relationship. Kinetic parameters obtained from three experiments were shown in Table 1, and V_{max} values (the maximum rate of PAH uptake), but not K_m values (the Michaelis constant), were significantly decreased to about 60% of control in the brush-border membrane vesicles treated with DEPC. These results suggest that the number of available transporters or their availability was decreased by DEPC treatment. This is comparable with the findings that the V_{max} values of various transport systems such as H^+/dipeptide cotransporters

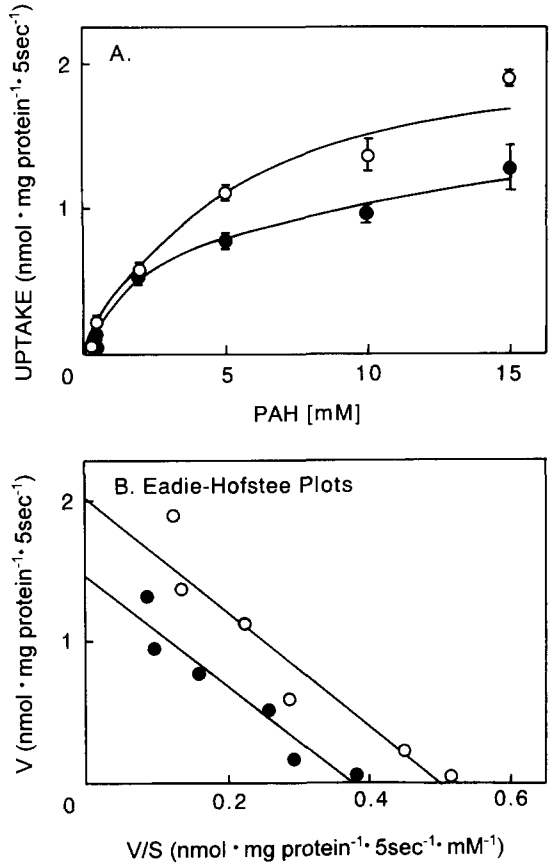

Fig. 5. Concentration dependence of potential-sensitive uptake of PAH by brush-border membrane vesicles. (A) Brush-border membrane vesicles were treated in the absence (○) or presence (●) of 0.1 mM DEPC. PAH uptake for 5 s at concentrations between 0.05 and 15 mM was measured in the presence or absence of valinomycin, and the latter was subtracted from the former to obtain the potential-sensitive component of the PAH uptake. Each point represents the mean of three determinations from a typical experiment. (B) Eadie-Hofstee plots of the data.

in the intestinal and renal brush-border membranes and H^+/organic cation antiporter in renal brush-border membrane were decreased by DEPC treatment [22–24].

Fig. 6 shows the effect of DEPC treatment in the presence of organic anions on PAH uptake. Brush-border membrane vesicles were treated with DEPC in the presence or absence of unlabeled PAH or

Table 1
Kinetic parameters of potential-sensitive PAH uptake in control and DEPC-treated brush-border membrane vesicles

Vesicles	K_m (mM)	V_{max} (nmol·mg protein^{-1}·min^{-1})
Control	4.9 ± 1.3	16.8 ± 4.1
DEPC-treated	4.5 ± 1.4	10.7 ± 3.5
	(90.7 ± 3.8)	(61.3 ± 7.4) **

Each value represents the mean ± S.E. of three experiments performed in three determinations. The values in parentheses represent the mean ± S.E. of the percentage of control.
** $P < 0.01$.

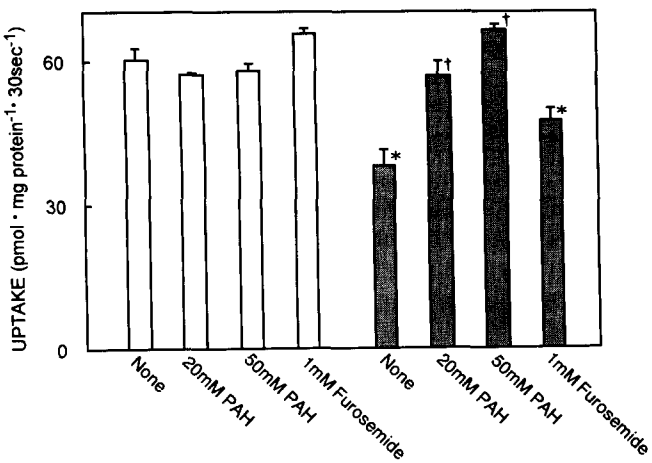

Fig. 6. Potential-sensitive uptake of PAH by brush-border membrane vesicles pretreated with DEPC in the presence of organic anions. Brush-border membrane vesicles were pretreated with (dotted column) or without (open column) 0.1 mM DEPC in the absence (None) or presence of either 20 or 50 mM PAH or 1 mM furosemide, and the potential-sensitive uptake of PAH for 30 s was measured. Each column represents the mean ± S.E. of six determinations from two experiments. * $P < 0.05$, significant difference from the uptake by control vesicles (open column, None). † $P < 0.05$, significant difference from the uptake by the vesicles treated with DEPC alone (dotted column, None) using one-way analysis of variance followed by Dunnett's t-test.

furosemide, and then potential-sensitive uptake of PAH was measured. Treatment of the membrane vesicles with these anions alone did not inhibit the PAH uptake, indicating that these organic anions were reversibly removed during the washing procedure after treatment. When PAH was present during the treatment of the vesicles with DEPC, PAH clearly attenuated the inhibitory effect of DEPC in a concentration-dependent manner. A similar result was obtained when furosemide was used, though it did not reach the significant difference. Under all experimental conditions, the equilibrium uptake of PAH was not altered (data not shown). Thus, the inhibitory effect of DEPC treatment on PAH uptake was protected by the presence of substrates for the transporter. These results indicate that amino acid residues modified by DEPC are located at or near the PAH binding site. Alternatively, the binding of substrate may affect the reactivity of the residues at sites other than the PAH binding site. DEPC preferentially reacts with histidine residues but it also reacts with sulfhydryl groups and tyrosine residues [25]. In order to obtain information about the amino acid residues modified by DEPC under our experimental conditions, we examined PAH uptake by the vesicles pretreated with DEPC and subsequently with hydroxylamine, a reagent which can reverse DEPC modification of histidine and tyrosine residues but not its modification of sulfhydryl groups [11,25]. However, PAH uptake by the vesicles treated with hydroxylamine alone was decreased, and therefore conclusive results could not be obtained (data not shown). We have previously studied the inhibitory effect of DEPC treatment on H^+/tetraethylammonium antiport in renal brush-border membrane vesicles [24]. In that study, the inhibition was considered to be due to the modification of histidine residues. In the present study, brush-border membrane vesicles were treated with DEPC under the same condition as before, and therefore we speculate that the inhibition of potential-sensitive PAH uptake by DEPC is probably due to the modification of histidine residues involved in the transport process.

In conclusion, a potential-sensitive transport of PAH but not PAH transport by an anion exchanger was inhibited by DEPC treatment in rat renal brush-border membrane vesicles. The amino acid residue, most likely a histidine residue, modified by DEPC may be located at or near the PAH binding site in the transporter. The present results indicate that potential-sensitive PAH transport and anion exchange are mediated by two structurally distinct transporters in the rat renal brush-border membrane. The distribution of these two PAH transporters along the nephron in the kidney, their physiological functions, and the precise role of various amino acid residues in these transporters have to be studied further.

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