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Inhibition of Dicarboxylate Transport by *p*-chloromercuribenzoic Acid (PCMB) in Plasma Membrane Vesicles of Rabbit Proximal Tubule

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= ABSTRACT =

Effect of a sulfhydryl reagent, *p*-chloromercuribenzoic acid (PCMB), on the transport of succinate was studied in brush border (BBMV) and basolateral (BLMV) membrane vesicles isolated from rabbit renal cortex. PCMB induced an irreversible inhibition of the Na^+-dependent succinate uptake in a dose-dependent manner with IC_{50} of 55 and 65 μM in BBMV and BLMV, respectively. The inhibitory effect of PCMB was prevented by a pretreatment of vesicles with dithiothreitol. PCMB did not increase Na^+ permeability at concentrations inhibiting succinate uptake. The PCMB inhibition of succinate uptake was due to a change in Vmax, but not in Km. When membrane vesicles were pretreated with PCMB in the presence of unlabelled succinate, the inhibitory effect was significantly reduced. In both BBMV and BLMV, succinate uptake was inhibited by various sulfhydryl reagents with the inhibitory potency of following order: $HgCl_2$ >DTNB>PCMBS>PCMB. These results suggest that sulfhydryl groups are essential for dicarboxylate transport and that they may be located at or near substrate binding sites of the transporters in renal brush border and basolateral membranes.

Key Words: Brush border membrane, Basolateral membrane, Dicarboxylate transport, Sulfhydryl group, PCMB, Rabbit proximal tubule.

INTRODUCTION

Krebs cycle intermediates, such as succinate, α-ketoglutarate, and citrate, are actively taken up by renal proximal tubular cells from lumen (Sheridan et al, 1983) as well as from peritubular side (Cohen and Wittman, 1963; Ullrich et al, 1984). Studies using isolated renal plasma membrane vesicles have demonstrated that the dicarboxylate transport in both brush-border (Kragh-Hansen et al, 1982; Wright, 1985) and basolateral (Wright & Wunz, 1987; Burckhardt, 1984; Lee et al, 1990) membranes is a Na^+-dependent process. The properties of the transport system in the two membranes are similar but the substrate affinity and the transport capacity are different (Kim et al, 1992).

Although the dicarboxylate transport system has been extensively studied, little information is available on its molecular structure. The precise structure of the transporter can only be delineated after its isolation and purification. However, the use of group-specific modifying reagents may provide insight into understanding the chemical nature of a transporter. Such approaches have been used in demonstrating the involvement of sulfhydryl (Klip et al, 1980; Krupka & Deves, 1988) and tyrosyl (Peerce & Wright, 1985) groups for the Na^+-glucose cotransporter and the in-

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volvement of carboxylic acid (Igarashi & Aronson, 1987; Kinsella et al, 1987) and histidine residues (Grillo & Aronson, 1986) for the renal Na^+-H^+ exchanger.

Histidyl group has been shown to be essential for the function of the Na^+-succinate cotransporter in renal brush border membrane (Bindslev & Wright, 1984). However, the role of sulfhydryl group in the dicarboxylate transport system has not been systematically studied, although several studies have implied that sulfhydryl group plays an important role in proximal tubular transports of various substrates (Klip et al, 1980; Sokol et al, 1986; Hori et al, 1987; Krupka & Deves, 1988; Pratt & Pedersen, 1989; Loghman-Adham, 1991).

Thus, in the present study, we determined the effect of *p*-chloromercuribenzoic acid (PCMB), sulfhydryl modifying reagent, on the transport of succinate in renal brush border (BBMV) and basolateral (BLMV) membrane vesicles. The results suggest that the sulfhydryl group is essential for the function of renal dicarboxylate transporter.

MATERIALS AND METHODS

Preparation of plasma membrane vesicles

BLMV and BBMV were simultaneously isolated from 1.5~2.5 kg New Zealand White rabbits of either sex by the Percoll-density gradient centrifugation and Mg^{2+}-precipitation method, as previously described (Lee et al, 1990).

The vesicles were suspended in the vesicle buffer, adjusted to yield a protein concentration of 6 mg/ml and stored at −70°C until use. Unless stated otherwise, the composition of vesicle buffer was 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.4). Prior to transport studies vesicles were preincubated at 37°C for 30 min to effectively load with appropriated buffer (Jung et al, 1989).

Transport studies

Uptake of succinate by vesicles was measured by a rapid filtration technique. Briefly, the reaction was initiated by adding membrane vesicles to the incubation medium (a 1 : 10 dilution of membrane vesicle suspension) containing [^{14}C] succinate at 25°C. The composition of the incubation medium was given in the figure legends. At the designated times, 100 μl aliquots were taken and quickly filtered under vacuum through Millipore filters (HAWP, 0.45 μm pore size) which were soaked overnight in distilled water. The filters were then washed with 5 ml of ice-cold stop solution containing 100 mM mannitol, 100 mM NaCl, 20 mM Hepes/Tris (pH 7.4) and 0.1 mM $HgCl_2$, and dissolved in 1.0 ml of methoxyethanol. After addition of 10 ml of scintillation cocktail, the amount of radioactivity taken up by vesicles was determined by liquid scintillation spectrometry (Packard Tricarb 300C). Nonspecific binding of substrate to membranes was determined by the same filtration procedure after vesicles were incubated in distilled water containing 0.1% deoxycholate and radioactive substrate.

Pretreatment of membrane vesicles with PCMB

Membranes were incubated at 20°C for 30 min with PCMB or its analogues. The mixture was diluted 20-fold with ice-cold vesicle buffer and centrifuged at 35,000 g for 60 min. The resulting pellet was then resuspended in the same buffer and used for transport measurements.

Measurement of Na^+ permeability

Na^+ permeability was measured by monitoring changes in fluorescence of diS-C_3-(5) (Gunther et al, 1984). Briefly, aliquots of membranes (100 μg of protein), which were loaded with 100 mM K^+-gluconate, 100 mM mannitol and 20 mM Hepes/Tris (pH 7.4), were diluted into 2 ml of media containing

100 mM Na^+-gluconate, 100 mM mannitol, 3 μM diS-C_3-(5), 1 μM valinomycin and 20 mM Hepes/Tris (pH 7.4). The fluorescence was recorded continuously at 25°C in a spectrofluorometer (Aminco, SPF-500C), equipped with an electronic stirring system and thermostated cuvette holder (excitation, 645 nm; emmision, 665 nm).

Marker enzyme assays

Na-K-ATPase activity was measured by the method of Jørgensen & Skou (1971) and alkaline phosphatase activity by the method of Linhardt & Walter (1963). Protein was determined according to Bradford (1976), using γ-globulin as a standard.

Calculations and statistical analysis

When required, the data were analyzed and fitted using the computer program ENZFITTER (Sigma Co, St. Louis, MO, U.S.A.). Student's *t*-test was used in statistical evaluation of the data and p-values less than 0.05 were considered significant.

Chemicals

[^{14}C] succinate (58.0 mCi/mmol) was purchased from the Amersham International (Amersham, UK). *p*-Chloromercuribenzoic acid (PCMB), *p*-chloromercuribenzene sulfonic acid (PCMBS), 5,5'-dithio-bis- (2-nitrobenzoic acid) (DTNB), dithiothreitol, valinomycin, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) were purchased from Sigma (St. Louis, MO, U.S.A.). 3,3'-Dipropylthiodicarbocyanine iodide [diS-C_3-(5)] was obtained from Molecular probes (Eugene, OR, U.S.A.).

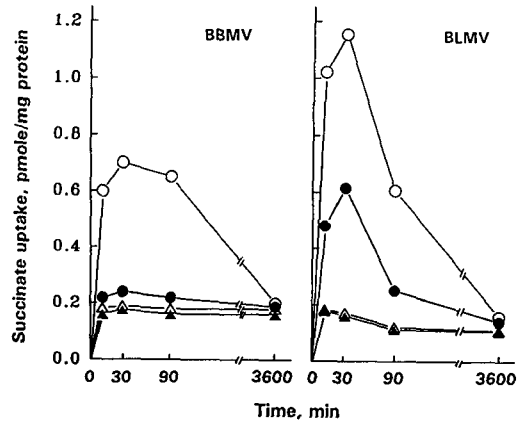

Fig. 1. Effect of PCMB on succinate uptake in BBMV and BLMV. Membrane vesicles were preloaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.4). Uptake of 5 μM [^{14}C] succinate was measured in buffer containing 100 mM mannitol, 100 mM NaCl (○, ●) or 100 mM KCl (△, ▲) and 20 mM Hepes/Tris (pH 7.4) in the presence (●, ▲) and absence (○, △) of 0.1 mM PCMB. Each point represents the mean of 2 determinations.

RESULTS

Effect of PCMB on succinate uptake

Fig. 1 shows the time courses of succinate uptake by BBMV and BLMV in the presence and absence of 0.1 mM PCMB. Uptake was measured in the presence and absence of an inwardly directed Na^+ gradient. The overshoot uptake of succinate in the presence of a Na^+ gradient was markedly reduced by PCMB. When Na^+ in the incubation medium was replaced by K^+, however, the succinate uptake was not altered by PCMB. The equilibrium values measured at 60 min of incubation were also identical in control and treated vesicles, indicating that PCMB did not affect vesicle integrity. A similar inhibitory effect by PCMB was observed in both BBMV and BLMV.

To examine the inhibitory effect of PCMB, the initial rate (10 sec) of Na^+-dependent succinate uptake was measured in the presence of various concentrations of PCMB. As shown in Fig. 2, Na^+-dependent succinate uptake in both BBMV and BLMV was inhibited by PCMB in a dose-dependent manner with IC_{50} value (the concentration for 50% inhibition)

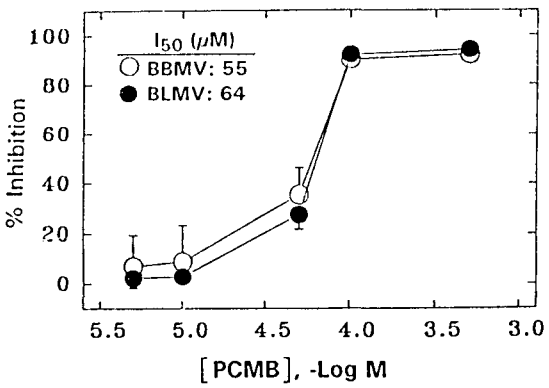

Fig. 2. Dose-dependence of the PCMB inhibition of succinate uptake in BBMV and BLMV. Membrane vesicles were preloaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris (pH 7.4). Uptake of 5 μM [^{14}C] succinate was measured for 10 sec in buffer containing 100 mM mannitol, 100 mM NaCl and 20 mM Hepes/Tris (pH 7.4) in the presence of various concentrations of PCMB. Each point represents the mean±SE of 5 experiments.

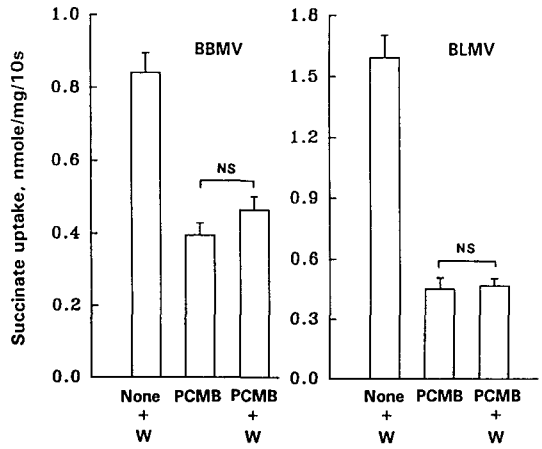

Fig. 3. Irreversibility of the inhibitory effect of PCMB on succinate uptake. Membrane vesicles were pretreated with 0.1 mM PCMB for 30 min and washed twice with PCMB-free vesicle buffer (W). Uptake of 5 μM [^{14}C] succinate was measured for 10 sec in buffer containing 100 mM mannitol, 100 mM NaCl and 20 mM Hepes/Tris (pH 7.4). Each point represents the mean±SE of 3 experiments. NS indicates nonsignificance.

of 55 and 64 μM, respectively.

To examine the reversibility of the PCMB effect, the membranes were treated with PCMB (0.1 mM) for 30 min, washed twice with PCMB-free vesicle buffer and then succinate uptake was determined. The results depicted in Fig. 3 indicate that in both BBMV and BLMV the PCMB effect was not reversed.

Effect of PCMB on Na^+ permeability

It has been reported that mercurials increase the inorganic cation permeability of the plasma membrane (Knauf & Rothstein, 1971; Will & Hopfer, 1979). In order to examine if the PCMB inhibition of Na^+-succinate cotransport was due to a facilitated dissipation of Na^+ gradient by PCMB rather than its specific interaction with the transport system. The effect of PCMB on the Na^+ permeability was determined using a potential-sensitive fluorescent dye, diS-C_3-(5). Fig. 4 shows the fluorescence changes observed in the control vesicles preloaded with 100 mM K^+-gluconate and suspended in a buffer containing 100 mM Na^+-gluconate and 3 μM diS-C_3-(5). Fluorescence was rapidly decreased by the generation of an inside-negative potential and then increased gradually by Na^+ influx; thus, the latter step reflects the permeability of Na^+ across the membrane. Such estimations of the Na^+ permeability in the control and various mercurial-treated vesicles are summarized in Table 1. As is seen, not only PCMB but also other mercurials, such as $HgCl_2$, DTNB, PCMBS, had no apparent effect on the Na^+ permeability of the BBMV. This, in turn, suggests that PCMB inhibition of Na^+-dependent succinate uptake was due to a direct interaction of the drug with the transport system.

Effect of dithiothreitol (DTT) on the PCMB inhibition

To assess whether the reaction of PCMB

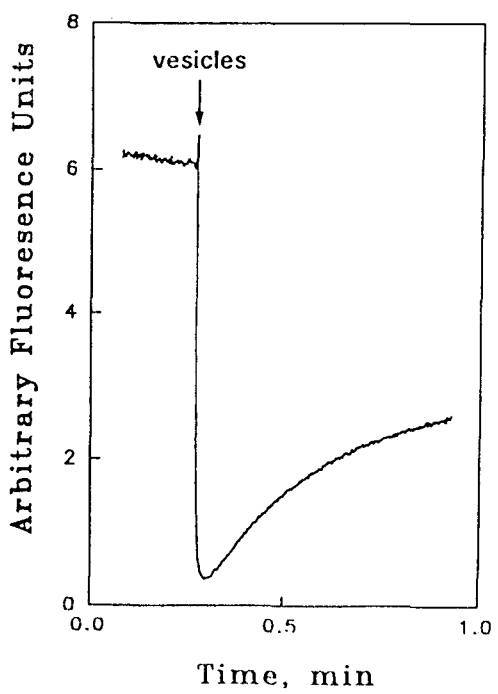

Fig. 4. A typical tracing of changes in fluorescence of dis-C_3-(5) after the addition of membrane vesicles (BBMV). Membranes, which were preloaded with 100 mM mannitol, 100 mM K^+ gluconate and 20 mM Hepes/Tris (pH 7.4), were diluted in buffer containing 100 mM mannitol, 100 mM Na^+-gluconate, 1 μM valinomycin, 3 μM diS-C_3-(5) and 20 mM Hepes/Tris (pH 7.4).

with the succinate transporter was specific for sulfhydryl groups, we examined the effect of DTT on the PCMB inhibition of succinate uptake and the results are summarized in Tabel 2. When PCMB was added to the membranes treated with 5 mM DTT, the succinate uptake was not significantly different from the control value obtained in the absence of PCMB. When DTT was added to the membranes pretreated with PCMB, however, the inhibition of succinate uptake was not reversed. These results indicate that PCMB inhibits irreversibly functional sulfhydryl groups of succinate transport system in brush border and basolateral membranes.

Table 1. Effect of sulfhydryl reagents on Na^+ permeability in BBMV

Reagents	Conc. (M)	Initial rate of return of fluorescence (△F/sec)
Control (no drug)		0.0029±0.00038
$HgCl_2$	6×10^{-5}	0.0027±0.00023
	1×10^{-4}	0.0025±0.00029
DTNB	6×10^{-5}	0.0026±0.00044
	1×10^{-4}	0.0021±0.00046
PCMBS	6×10^{-5}	0.0028±0.00022
	1×10^{-4}	0.0025±0.00024
PCMB	6×10^{-5}	0.0026±0.00010
	1×10^{-4}	0.0026±0.00011

Membranes were pretreated with sulfhydryl reagents and washed twice with reagent-free vesicle buffer. A change in fluorescence of diS-C_3-(5) was measured in the control and reagent-pretreated membranes as described in Fig. 4. In these experiments, the initial rate of return of fluorescence (△F/sec) reflects Na^+ permeability. Data are the mean±SE of four experiments.

Effect of PCMB on kinetics of Na^+-dependent succinate uptake

To elucidate the nature of the PCMB inhibition, the effect of PCMB pretreatment on the kinetics of succinate uptake was examined. The initial rate of Na^+-dependent succinate uptake was analyzed using a computerized model of Michaelis-Menten kinetics. Fig. 5 depicts Na^+-dependent succinate uptakes by BBMV and BLMV as a function of succinate concentration. The PCMB pretreatment decreased maximum velocity (Vmax) from 70.21±0.96 to 46.80±7.44 in BBMV and from 39.02±3.11 to 25.92±1.58 nmole/mg/min in BLMV. However, the value of Km was not significantly altered in both membranes. These results suggest that PCMB decreased the number of transporter or turnover rate of an

Table 2. Effect of dithiothreitol (DTT) on succinate uptake

Pretreatment	Uptake (nmole/mg protein)	
	BBMV	BLMV
Control	0.841±0.055	1.353±0.110
+PCMB	0.395±0.034**	0.453±0.055**
Addition of DTT after PCMB treatment	0.403±0.034**	0.624±0.042**
Addition of DTT before PCMB treatment	0.739±0.085	1.685±0.163
+DDT	0.785±0.090	1.455±0.061

Membrane vesicles were pretreated with 0.1 mM PCMB for 30 min after or before the addition of 5 mM DTT and washed with PCMB-free vesicle buffer. Succinate uptake was measured for 10s in buffer containing 100 mM mannitol, 100 mM NaCl and 20 mM Hepes/Tris (pH 7.4). Data are the mean±SE of 3 experiments.
** $P<0.01$ vs. control.

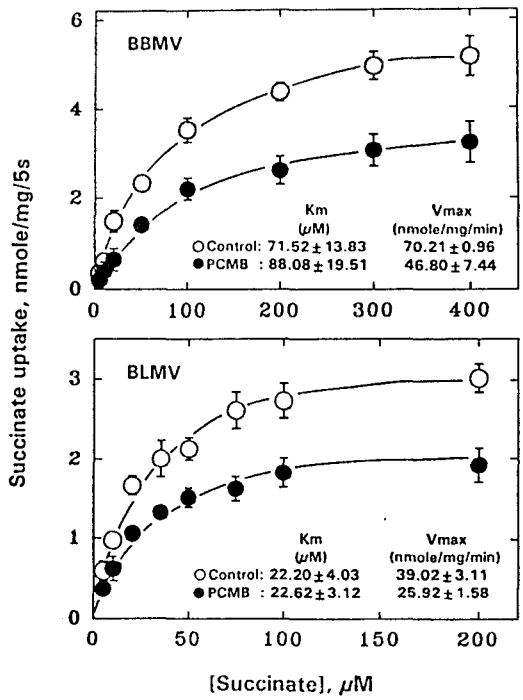

Fig. 5. Effect of PCMB on kinetics of Na^+-dependent succinate uptake in BBMV and BLMV. Membrane vesicles were pretreated with 0.1 mM PCMB for 30 min. Uptake was measured in the control and PCMB-treated membranes for 5 sec in buffer containing 100 mM mannitol, 100 mM NaCl, and 20 mM Hepes/Tris (pH 7.4) in the presence of various concentrations of [^{14}C] succinate. Each point represents the mean±SE of 5 experiments.

individual transporter without an effect on substrate affinity.

Effect of substrate and Na^+ on the PCMB inhibition

We next examined if the substrate or Na^+ would protect the transport system from PCMB inhibition. The results are shown in Table 3. When the membranes were pretreated with PCMB in the presence of unlabelled succinate (5 mM), the inhibition was significantly reduced compared with those pretreated in the absence of succinate. However, the inhibition of succinate uptake by PCMB pretreatment was not latered by the presence of Na^+ in the preincubation medium.

Effects of various sulfhydryl reagents on Na^+-dependent succinate uptake

In the next series of experiments, effects of various sulfhydryl reagents on the initial rate of Na^+-dependent succinate uptake were compared. As shown in Fig. 6, $HgCl_2$, DTNB and PCMBS also inhibited succinate uptake in a dose-dependent fashion. The IC_{50} values increased in the following order: $HgCl_2$< DTNB<PCMBS<PCMB in both membranes.

Since the solute uptake by a vesicle is directly related to the vesicular volume, the effect of sulfhydryl reagent on the vesicular volume was estimated from the equilibrium value (60 min uptake). Fig. 7 depicts the results. In both BBMV and BLMV, $HgCl_2$, DTNB and PCMBS reduced the equilibrium

Table 3. Effect of substrate and Na^+ on the PCMB inhibition of succinate uptake

Treatment	BBMV		BLMV	
	Uptake (nmole/mg • 10s)	Inhibition (%)	Uptake (nmole/mg • 10s)	Inhibition (%)
Control	0.729±0.036		1.408±0.076	
+PCMB	0.229±0.032	68.50±5.48	0.435±0.056	69.26±6.34
+SA	0.708±0.030		1.327±0.090	
+SA+PCMB	0.473±0.021	33.12±3.86*	0.714±0.024	43.11±3.16*
$+Na^+$	0.720±0.078		1.266±0.066	
$+Na^+$+PCMB	0.316±0.062	56.26±6.22	0.413±0.037	66.32±4.72

Membrane vesicles were treated with 0.1 mM PCMB in the presence of 5 mM unlabelled succinate (SA) or 100 mM NaCl (Na^+). After washing the membranes, uptake of 5 μM [^{14}C] succinate was measured for 10s in buffer containing 100 mM mannitol, 100 mM NaCl and 20 mM Hepes/Tris (pH 7.4). Data are the mean±SE of 5 experiments. * $P<0.01$ vs. PCMB inhibition in the control medium.

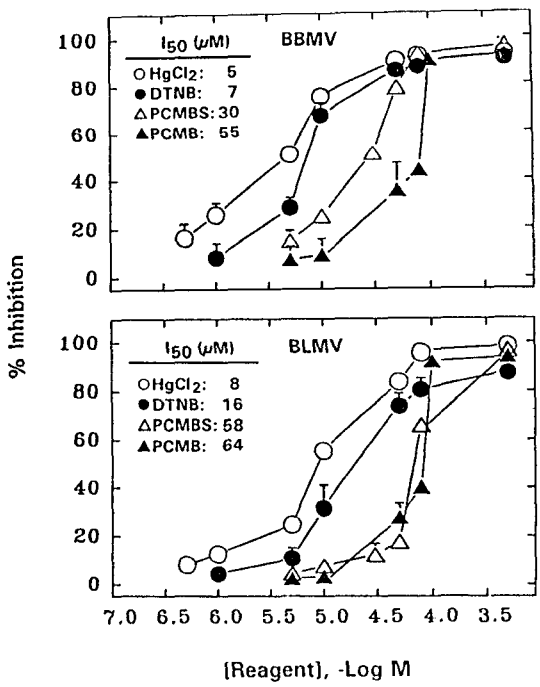

Fig. 6. Effects of various sulfhydryl reagents on succinate uptake. Uptake of 5 μM [^{14}C] succinate was measured for 10 sec as described in Fig. 2. Each point represents the mean±SE of 4 experiments.

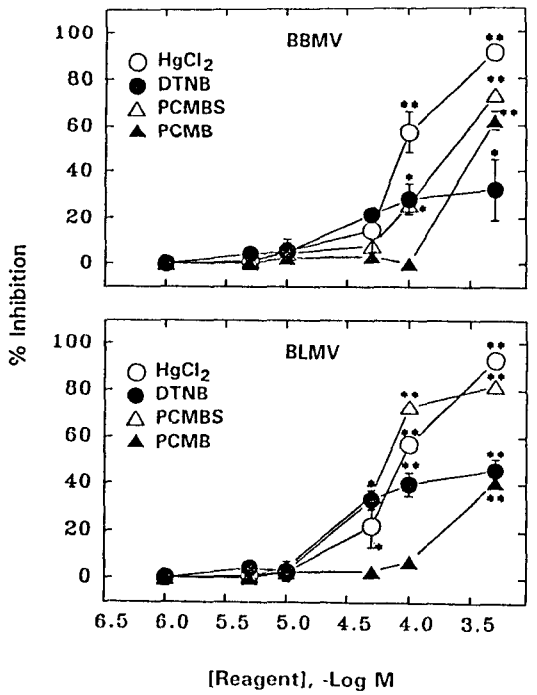

*Fig. 7. Effects of various sulfhydryl reagents on the equilibrium uptake of succinate. Uptake of 5 μM [^{14}C] succinate was measured for 60 min as described in Fig. 2. Each point represents the mean ±SE of 4 experiments. * $P<0.05$; ** $P<0.01$*

value significantly at high concentrations, but not at concentrations near IC_{50}.

DISCUSSION

The present study demonstrated that PCMB, a sulfhydryl-group modifying reagent, inhibits irreversibly the Na^+-dependent succinate uptake in BBMV and BLMV. Such an effect of PCMB could be from a direct interaction of the drug with the carrier or from an enhanced dissipation of the Na^+ gradient, a driving force of a Na^+-dependent solute transport. Kone et al (1990) reported that mercurials cause rapid, dramatic changes in membrane ionic permeability of the proximal tubule. Will & Hopfer (1979) also demonstrated that the inhibition of Na^+-dependent uptake of amino acid and glucose by PCMB in intestinal BBMV is secondary to an increase in the membrane Na^+ conductance. In this study, however, the Na^+ permeability of the vesicle was not altered by various sulfhydryl reagents at the concentration for 50% inhibition (Fig. 4).

In this study, the PCMB inhibition of succinate uptake was completely blocked by a pretreatment of vesicles with DTT. When membrane vesicles were pretreated with PCMB, however, the inhibition was not reversed by the addition of DTT. These results indicate that the inhibition of succinate uptake by PCMB is due to a specific interaction of the drug with essential sulfhydryl groups in the dicarboxylate carrier protein.

In kinetic analysis of succinate uptake by BBMV and BLMV, the Vmax was decreased, but the apparent Km was not changed in the PCMB-treated membranes. These results indicate that the inhibition of succinate transport by PCMB is noncompetitive in nature.

To determine whether sulfhydryl residues are located at the binding site of dicarboxylates, the effect of PCMB pretreatment on succinate uptake was examined in the presence of unlabelled succinate. If sulfhydryl residues reside at the substrate binding site, succinate could bind to these residues and thus prevent them from PCMB interaction. We observed that the PCMB inhibition of [^{14}C] succinate uptake was significantly reduced in the presence of 5 mM unlabelled succinate, suggesting that the sulfhydryl residues may be located at the binding site of the dicarboxylate transporter. In contrast, the PCMB inhibition was not changed by the presence of 100 mM Na^+ in the preincubation medium. Although these results suggest that the modified sulfhydryl groups are located at the substrate binding site, the data do not exclude the possibility that the presence of a high concentration of substrate induces the conformational changes in the transporter molecule and consequently prevent the interaction of sulfhydryl group and PCMB.

Various mercurials reduced the vesicle volume, estimated from the equilibrium uptake, at high concentrations, in accordance with other reports (Will & Hopfer, 1979; Loghman-Adham, 1991). However, Na^+-dependent succinate uptake was inhibited at inhibitor concentrations that did not produce significant changes in vesicular volume. Therefore, the inhibitions of succinate uptake by various mercurials were not associated with the vesicular volume change.

In this study, the effect of sulfhydryl reagents on succinate uptake was similar in BBMV and BLMV. This suggests that the dicarboxylate transporters of both membranes may be similar in molecular structure.

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