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Effects of Ethanol on Na^+-dependent Solute Uptake in Rabbit Renal Brush-Border Membrane Vesicles

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This study was undertaken to examine the effect of ethanol on Na^+-dependent transport systems (glucose, phosphate, and dicarboxylate) in renal brush-border membrane vesicles (BBMV). Ethanol inhibited Na^+-dependent uptakes of glucose, phosphate, and succinate in a dose-dependent manner, but not the uptakes of Na^+-independent. The H^+/TEA antiport was reduced by 8% ethanol. Kinetic analysis showed that ethanol caused a decrease in V_{max} of three transport systems, leaving K_m values unchanged. Ethanol decreased phlorizin binding, which was closely correlated with the decrease in V_{max} of Na^+-glucose uptake. These results indicate that ethanol inhibits Na^+-dependent uptakes of glucose, phosphate, and dicaboxylate and that the reduction in V_{max} of Na^+-glucose uptake is caused by a decrease in the number of active carrier proteins in the membrane.

Key Words: Ethanol, Phosphate uptake, Glucose uptake, Brush-border membrane vesicles, Rabbit kidney

INTRODUCTION

Effects of benzyl alcohol, a membrane fluidizer, on Na^+-dependent phosphate (Na^+-P_i) uptake in cultured proximal tubular cells are different from those on Na^+-dependent glucose (Na^+-glucose) and amino acid (Na^+-alanine) uptakes. Exposure of cells to benzyl alcohol causes stimulation of Na^+-P_i uptake and inhibition of Na^+-glucose uptake without any changes in Na^+-dependent alanine uptake (Friedlander et al, 1988; Friedlander et al, 1990). These results suggest that changes in membrane fluidity affect Na^+-dependent transport systems in renal cells differently. Similar results were also reported in isolated renal brush-border membrane vesicles (BBMV) (Yusufi et al, 1989). Certain *n*-alcohols including ethanol, like benzyl alcohol, have been shown to modulate Na^+-glucose uptake by altering the fluidity in intestinal (Fernandez et al, 1984) and renal (Elgavish & Elgavish, 1985) BBMV, although controversial data are reported (Tillotson et al, 1981). However, effects of ethanol on other Na^+-dependent transport systems such as Na^+-Pi and Na^+-dicarboxylate uptake in renal BBMV have not been explored.

Studies with renal BBMV have shown that ethanol-induced inhibition of Na^+-glucose uptake is attributed to dissipation of the Na^+ gradient resulting from an increase in Na^+ permeability (Fernandez et al, 1984; Elgavish & Elgavish, 1985; Parenti et al, 1991). Similar results have been reported in intestinal brush-border membrane vesicles (Tillotson et al, 1981). Alternatively, the decrease in V_{max} in ethanol-treated renal BBMV could be due to a reduction in the number of Na^+-dependent transporters present in the membrane. It has been reported that benzyl alcohol induces a decrease in phlorizin binding in renal BBMV, suggesting that changes in membrane fluidity cause a decrease in the number of active transport carriers (Yusufi et al, 1989). Therefore, effect of ethanol on V_{max} of Na^+-dependent uptakes may not be completely accounted for by an increase in Na^+ permeability.

The present study was undertaken in BBMV isolated from rabbit renal cortex to examine (1) the effect of ethanol on Na^+-dependent uptakes of glucose, phosphate, and succinate, and (2) determine

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whether ethanol-induced reduction in V_{max} results from changes in the number of carrier proteins present in the membrane. Alterations in the number of Na^+-glucose and Na^+-P_i transporters were estimated by the phlorizin and phosphoformic acid (PFA) binding, respectively. Phlorizin and PFA bindings have been used extensively for analysis of Na^+-glucose (Aronson, 1978; Molitoris & Kinne, 1987) and Na^+-Pi transporters (Szczepanska et al, 1987; Hoppe et al, 1991) in renal BBMV.

METHODS

Preparation of plasma membrane vesicles

BBMV were isolated from the kidney cortex of New Zealand White rabbits by the Mg^{2+}-precipitation method according to the procedure of Booth & Kenny (1974). Briefly, cortical tissues were homogenized in 10 mM mannitol and 2 mM Tris/HCl (pH 7.10) at 4°C. The homogenate was centrifuged for 2 min at 200 g to remove unbroken cells before proceeding to the Mg^{2+} treatment. Solid $MgCl_2$. $6H_2O$ was added to the homogenate to give a concentration of 10 mM, stirred for 15 min in an ice bath, and then centrifuged for 12 min at 1,500 g. The supernatant was saved and centrifuged for 12 min at 15,000 g. Pale-pink layer on top of pellet was removed and resuspended in 10 mM mannitol and 2 mM Tris/HCl (pH 7.10). Solid $MgCl_2$. $6H_2O$ was added, like before, to the suspension, stirred for 15 min, centrifuged, and then the pellet was resuspended in the vesicle buffer. The composition of vesicle buffer is given in figure legends. Protein was determined according to Bradford (1976), using γ-globulin as a standard. The purity of BBBV was evaluated by measuring the specific activities of membrane marker enzymes. Alkaline phosphatase activity was determined as described by Linhardt & Walter (1963) and was used as a BBM marker. Na^+-K^+-ATPase activity was measured according to Jörgensen & Skou (1971) and was used as a basolateral membrane marker. The enrichment of alkaline phosphatase activity and Na^+-K^+-ATPase activity in BBMV was approximately 11 and 1.4, respectively.

Transport studies in membrane vesicles

BBMV were pretreated with ethanol for 10 min at 25°C, and the same concentration of ethanol was added to incubation medium. The uptake of substrates by BBMV were determined 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 appropriate concentration of [^{14}C]-D-glucose, $K_2H^{32}PO_4$, [^{14}C]-succinate, or [^{14}C]-TEA at 25°C. The composition of the incubation medium is given in figure legends. At the designated times, 100 μl aliquots were taken and quickly filtered in vacuum through Millipore filters (HAWP, 0.45 μm pore size) which had been soaked overnight in distilled water. The filters were then washed with 5 ml of ice-cold stop solution comprising the identical composition to the incubation medium but without substrate, and dissolved in 1.0 ml of methoxyethanol. After the addition of 10 ml of scintillation cocktail, the amount of radioactivity taken up by vesicles was determined by liquid scintillation spectrometry (Packard Tricarb 300C). Na^+-dependent uptake was determined by subtracting the uptake in the absence of Na^+ (replaced by K^+) from that in the presence of Na^+. Nonspecific binding of radioactive substrate to the plasma membrane was determined by incubating vesicles in transport buffer containing 0.1% deoxycholate and radiolabeled substrates. All uptake data were corrected for nonspecific binding.

[^{3}H]Phlorizin and [^{14}C]PFA binding assay

The binding of [^{3}H]phlorizin or [^{14}C]PFA was measured using a rapid filtration technique. BBMV were incubated with various concentrations of [^{3}H]phlorizin or 1 mM [^{14}C]PFA for 10 min at 25°C in the medium containing 100 mM mannitol, 100 mM NaCl or KCl, 20 mM Hepes/Tris (pH 7.4) in the presence and absence of 5% ethanol. After incubation, the incubation mixture was quickly filtered under vacuum through Millipore filters, and the filters were prepared for measurement of radioactivity retained on the filter disc as described in uptake experiments. Na^+-dependent binding was calculated from the difference between uptakes in the presence and absence (KCl) of Na^+.

Materials

[^{14}C]-D-glucose, $K_2H^{32}PO_4$, [^{14}C]-succinate, [^{14}C]-tetraethylammonium (TEA), [^{3}H]phlorizin, and [^{14}C]

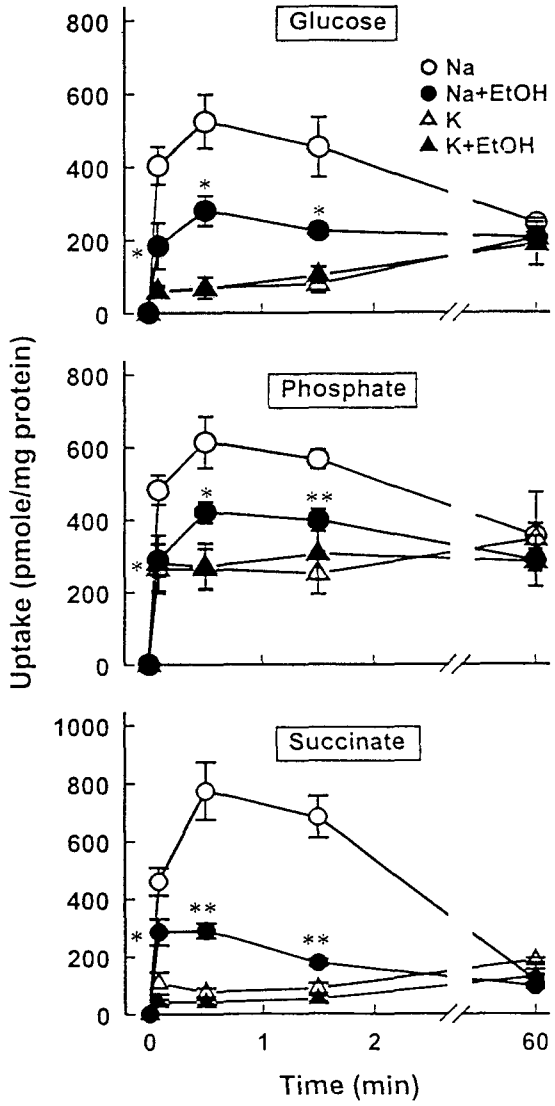

Fig. 1. Effect of ethanol on Na^+-dependent and independent uptakes of glucose, phosphate and succinate in renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.4), and pretreated with 5% ethanol or equal volume of vesicle buffer for 10 min at 25°C. The uptake was measured at 25°C in a buffer containing 100 mM Mannitol, 100 mM NaCl (○,●) or KCl (△,▲), 20 mM Hepes/Tris (pH 7.4) in the presence (●,▲) and absence (○,△) of 5% ethanol. The substrate concentration was 50 μM for [^{14}C]-D-glucose, and 5 μM for $H_3{}^{32}PO_4$ and [^{14}C]-succinate. Data are mean±SE of 4-5 experiments. *$p<0.05$ compared with control NaCl.

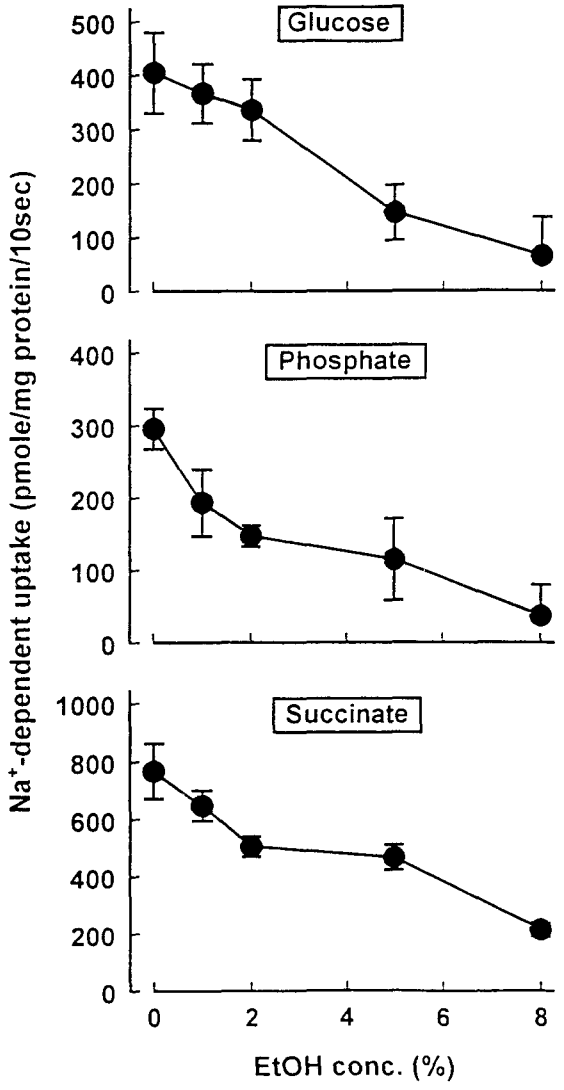

Fig. 2. The uptakes of glucose, phosphate and succinate as a function of ethanol concentration in renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.4), and pretreated with various concentrations of ethanol for 10 min at 25°C. The uptake was measured for 10 sec at 25°C in a buffer containing 100 mM Mannitol, 100 mM NaCl, 20 mM Hepes/Tris (pH 7.4) in the presence of ethanol. The substrate concentration was 50 μM for [^{14}C]-D-glucose, and 5 μM for $H_3{}^{32}PO_4$ and [^{14}C]-succinate. Data are mean±SE of 3-4 experiments.

phosphoformic acid (PFA) were purchased from the Amersham International (Amersham, UK). All the other chemicals were of the highest quality available.

Statistical analysis

The data are expressed as mean±SE. Comparison between the two groups was made by unpaired *t* test. Multiple group comparison was done using one-way

analysis of variance followed by Tukey *post hoc* test. The differences with $P<0.05$ were considered statistically significant.

RESULTS

In order to examine the effect of ethanol on Na^+-dependent transport systems in BBMV, time courses of glucose, phosphate, and succinate uptake were measured in the presence and absence of Na^+ in control and ethanol-treated of BBMV. The results are described in Fig. 1. For control BBMV, imposition of an inwardly directed Na^+ gradient revealed a transient overshoot above the equilibrium level. Treatment of BBMV with 5% ethanol resulted in a significant inhibition of the uptakes in the presence of a Na^+ gradient at all times, except at the equilibrium value. By contrast, ethanol did not alter the uptakes in the absence of Na^+, indicating that ethanol did not cause alterations in the passive permeability of the BBM to these substrates.

When BBMV were treated with various concentrations of ethanol, the uptakes of three substrates were inhibited in a dose-dependent manner (Fig. 2).

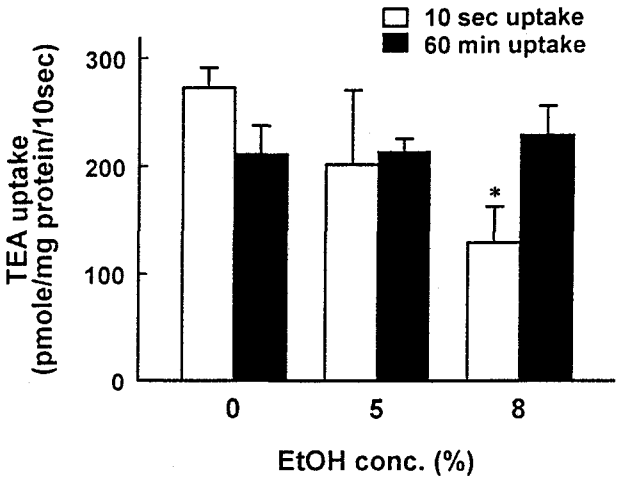

Fig. 3. Effect of ethanol on H^+/TEA antiport in renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Mes/Tris (pH 6.0), and pretreated with 5 and 8% ethanol or equal volume of vesicle buffer (0 ethanol) for 10 min at 25°C. The uptake was measured for 10 sec at 25°C in a buffer containing 50 μM [^{14}C]-TEA, 100 mM Mannitol, 100 mM KCl, 20 mM Hepes/Tris (pH 7.4) in the presence or absence of ethanol. Data are mean±SE of 3 experiments. *$p<0.05$ compared with 0 ethanol.

The I_{50} (the concentration for 50% inhibition) of glucose, phosphate, and succinate was approximately 4.2, 2.0, and 6.0%, respectively.

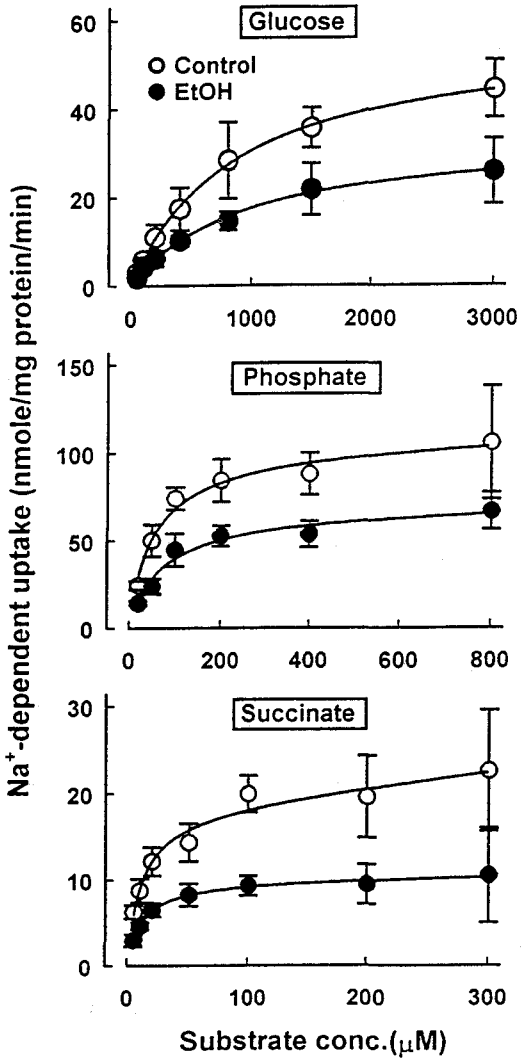

Fig. 4. Concentration dependency of Na^+-dependent glucose, phosphate and succinate uptake in control (○) and ethanol-treated (●) renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.4), and pretreated with 5% ethanol or equal volume of vesicle buffer (Control) for 10 min at 25°C. The uptake was measured for 7 sec at 25°C in a buffer containing 100 mM Mannitol, 100 mM NaCl or KCl, 20 mM Hepes/Tris (pH 7.4) in the presence or absence of 5% ethanol. The substrate concentration was 50 μM for [^{14}C]-D-glucose, and 5 M for $H_3{}^{32}PO_4$ and [^{14}C]-succinate. Na^+-dependent uptake was calculated by subtracting the uptake in the KCl medium from that in the NaCl medium. Data are mean±SE of 4-5 experiments.

To evaluate if ethanol inhibits a transport system driven by a solute other than Na^+, the effect of ethanol on H^+/TEA antiport was examined. As shown in Fig. 3, ethanol at 8% slightly inhibited H^+/TEA antiport, without any effect on the equilibrium value (60-min uptake). These results suggest that the H^+-driven transport system is more resistant to ethanol than Na^+-dependent systems.

To elucidate the nature of the ethanol inhibition, the effect of ethanol on the kinetics of Na^+-dependent transport systems was examined. Membrane vesicles were treated with 5% ethanol for 10 min. The initial (7 sec) rate of uptake was determined as a function of the external substrate concentration in the presence or absence of a Na^+ gradient. The Na^+-dependent component was calculated by subtraction. The results are summarized in Fig. 4. Hofstee transformation of the data (Fig. 5) clearly showed a noncompetitive inhibition pattern, the V_{max} of the Na^+-cotransporters was reduced by treatment of ethanol, but K_m was not altered (Table 1).

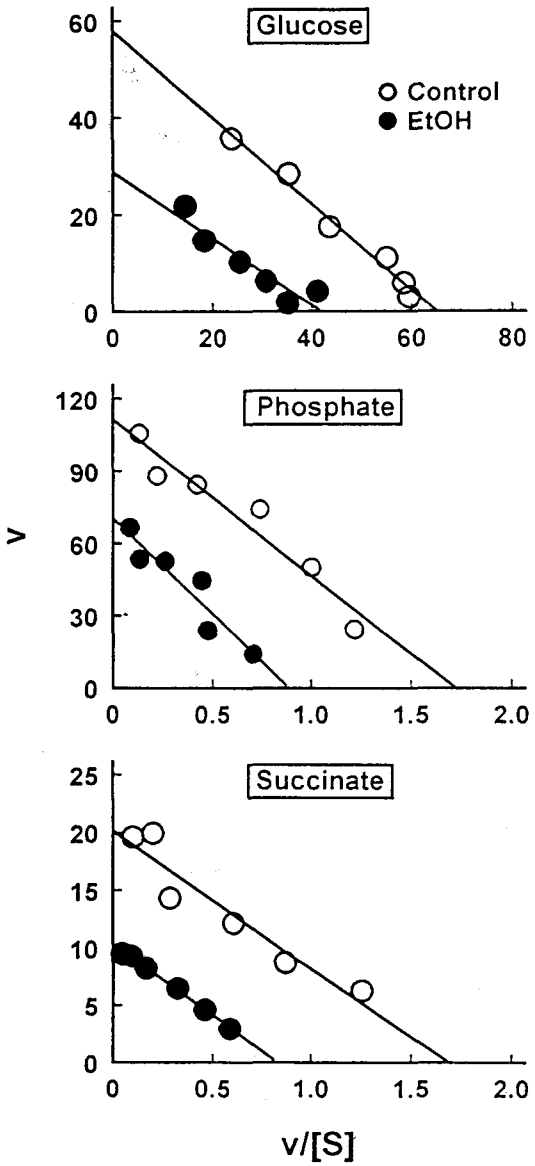

Fig. 5. Hofstee plots of the data in Fig. 4. The regression lines were fitted by least-squares analysis. In this plot, the intercept of the line with Y-axis represents V_{max} and the slope represents $-K_m$.

In the last series of experiments, we determined whether ethanol-induced reduction in V_{max} of Na^+-cotransporters is attributed to a loss of carrier proteins present in the membrane. Phlorizin is a well known nontransported inhibitor of renal Na^+-glucose transport, which competes with glucose for its binding sites on the carrier (Turner & Silverman, 1981; Turner & Moran, 1982a; Turner & Moran, 1982b; Kim & Park, 1995). PFA is a specific competitive inhibitor of Na^+-P_i cotransport and has been employed as a probe for studies of this transport system (Szczepanska et al, 1987; Yusufi et al, 1989; Hoppe et al, 1991). Thus, we examined effect of ethanol on phlorizin and PFA binding to BBMV in the presence and absence of an Na^+ gradient. The Na^+-dependent component was calculated by subtraction. As shown in Fig. 6A, the binding was increased curvilinearly as a function of phlorizin concentration, and it was significantly reduced by 5% ethanol. Scatchard plots of the data (Fig. 6B) indicated that ethanol reduced the number of total binding sites from 220.32 to 149.14, but the dissociation constant was not altered

Table 1. Effects of ethanol on kinetic parameters of Na^+-dependent uptakes in renal BBMV

Substrates	Treatment	V_{max} (nmole/mg/min)	K_m (mM)
Glucose	Control	56.96±7.23	0.86±0.13
	Ethanol	34.03±10.16	0.93±0.33
Phosphate	Control	101.96±15.16	0.05±0.02
	Ethanol	66.08±16.56	0.07±0.04
Succinate	Control	18.23±2.30	0.011±0.004
	Ethanol	9.95±0.44	0.011±0.001

The values of V_{max}, but not K_m, for three transport systems in ethanol-treated BBMV were significantly different ($p<0.05$) from the respective control.

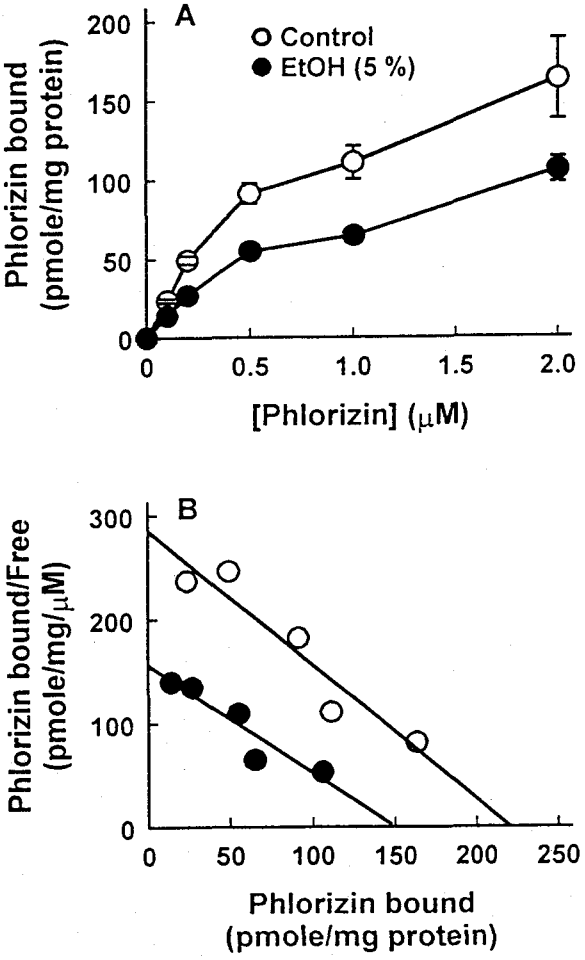

Fig. 6. A, Na^+-dependent component of [^{3}H]phlorizin binding as a function of phlorizin concentration in control (○) and ethanol-treated (●) renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.4), and incubated for 10 min at 25°C in a buffer containing 100 mM Mannitol, 100 mM NaCl or KCl, 20 mM Hepes/Tris (pH 7.4), and 0.1~1 μM [^{3}H]phlorizin in the presence or absence of 5% ethanol. Na^+-dependent component was calculated by subtracting the uptake in the KCl medium from that in the NaCl medium. Data are mean±SE of 3 experiments. B, Scatchard plots of data in Fig. A. In ethanol-treated BBMV, the number of binding sites decreased from 220.32 of control to 74.55, but the dissociation constant was not altered.

(1.30 vs. 1.05). Similarly, PFA binding was also decreased by 5% ethanol (Fig. 7).

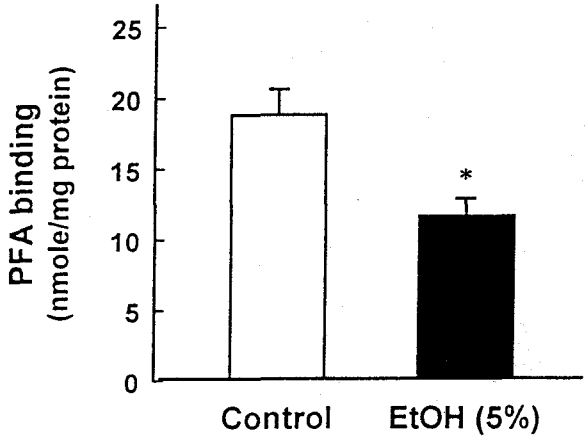

Fig. 7. A, Na^+-dependent component of [^{14}C]PFA binding in control and ethanol-treated renal BBMV. Membrane vesicles were loaded with 100 mM mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.4), and incubated for 10 min at 25°C in a buffer containing 100 mM Mannitol, 100 mM NaCl or KCl, 20 mM Hepes/Tris (pH 7.4), and 1 mM [^{14}C]PFA in the presence or absence of 5% ethanol. Na^+-dependent component was calculated by subtracting the uptake in the KCl medium from that in the NaCl medium. Data are mean±SE of 3 experiments. *$p<0.05$ compared with control.

DISCUSSION

The present study demonstrates that ethanol inhibits Na^+-dependent uptakes of glucose, phosphate, and succinate in renal BBMV. The inhibitory potencies of ethanol on these three transport systems were similar. Such results are different from those observed with benzyl alcohol in renal cells (Friedlander et al, 1988; Friedlander et al, 1990). In the studies which used, it stimulated Na^+-P_i uptake and inhibited Na^+-dependent glucose uptake in LLC-PK_1 cells and primary cultured proximal tubular cells. Although ethanol, like benzyl alcohol, increases membrane fluidity (Chin et al, 1979; Fernandez et al, 1984; Ives & Verkman, 1985), its effect on transport systems may be not solely due to alterations in the membrane fluidity. In fact, Tilloston et al (1981) reported that the inhibitory effect of ethanol on Na^+-glucose uptake is not attributed to changes in membrane fluidity in intestinal BBMV. Studies with isolated renal BBMV also demonstrated that benzyl alcohol *in vitro* treatment stimulated Na^+-P_i uptake without any effect on Na^+-succinate uptake (Yusufi et al, 1989), unlike ethanol in the present study. Interestingly, Na^+-P_i uptake by BBMV is stimulated by an increase in membrane fluidity resulting from thyroid hormone *in vivo* treatment (Yusufi et al, 1989), but the uptake is reduced by an increase in membrane fluidity resulting

from gentamicin *in vivo* treatment (Levi & Cronin, 1990).

A number of studies have demonstrated that ethanol-induced decrease in Na^+-glucose cotransport results from the dissipation of Na^+ gradient by increase in Na^+ permeability (Fernandez et al, 1984; Elgavish & Elgavish, 1985; Parenti et al, 1991). In the present study, although ethanol did not affect Na^+-dependent uptakes in the absence of Na^+ gradient, 8% ethanol significantly decreased H^+/TEA antiport, a transport system driven by an H^+ gradient other than Na^+ gradient. Since in the present study we did not determine the H^+ permeability in ethanol-treated BBMV, we could not exclude the possibility that ethanol causes the dissipation of H^+ gradient and thus inhibits H^+/TEA antiport. However, several investigators have proposed that the ethanol effect on membrane transport systems is caused by a direct interaction with carrier proteins (Deves & Krupka, 1990; Parenti et al, 1991).

Kinetic analyses showed that ethanol reduces V_{max} without any changes in K_m value in all the transport systems (Fig. 4). The V_{max} of Na^+-dependent transport systems could be modulated by alterations in membrane fluidity. However, different responses of three transport systems, Na^+-P_i, Na^+-glucose and Na^+-succinate cotransports, to a membrane fluidizer benzyl alcohol have been reported in renal BBMV (Yusufi et al, 1989). Exposure of isolated BBMV to benzyl alcohol stimulates Na^+-P_i uptake, while decreasing Na^+-glucose uptake and leaving Na^+-succinate uptake unchanged. These results are different from our data on ethanol effects. Thus, reduction of V_{max} in ethanol-treated BBMV may not be attributed to changes in membrane fluidity.

Alternatively, V_{max} could be modulated by changes in the number of transport carrier in the membrane. Alterations in the number of active carriers for Na^+-glucose have been estimated by using the binding of phlorizin. This drug is a well known competitive inhibitor of Na^+-glucose transport (Turner & Silverman, 1981; Turner & Moran, 1982a; Turner & Moran, 1982b; Kim & Park, 1995), which has been used extensively over many years for the analysis of Na^+-glucose cotransporter in BBM of renal and intestinal epithelial cells (Aronson, 1978; Toggenburger et al, 1982; Molitoris & Kinne, 1987). PFA competitively inhibits Na^+-P_i transport across BBM of proximal tubules both *in vitro* (Szczepanska et al, 1989; Hoppe et al, 1991) and *in vivo* (Szczepanska et al, 1986). Studies with renal BBMV demonstrated that PFA exerts its inhibitory effect by binding onto the same locus on the luminal surface of BBM where P_i and Na^+ form a ternary complex with Na^+-P_i cotransporter (Szczepanska et al, 1987; Szczepanska et al, 1989). Thus, PFA has been used as a probe for studies of Na^+-P_i cotransporter in BBM (Yusufi et al, 1989; Levi & Cronin, 1990; Hoppe et al, 1991), in a manner similar to phlorizin. Using these probes, we determined if ethanol decreased the number of active carriers for glucose and phosphate transport. The results obtained from the present study showed that ethanol decreases Na^+-dependent phlorizin and PFA binding, suggesting that the decrease in V_{max} by ethanol results from a decrease in the number of transporters in the membrane. Although, in the present study, ethanol did not cause an increase in the passive permeability of BBMV to glucose, P_i, and succinate, it would be inappropriate to extrapolate this observation to the effect of ethanol on Na^+ permeation. Therefore, we are unable to rule out the possibility that the ethanol-induced reduction of Na^+-dependent uptakes was, at least in part, related to an alteration in permeability of the BBM to Na^+.

In conclusion, ethanol decreased Na^+-dependent uptakes of glucose, P_i, and dicarboxylate. Reduction in V_{max} of Na^+-glucose uptake in ethanol-treated BBMV was due to a decrease in the number of operative carrier proteins in the membrane. However, it is not clear whether alterations in the number of active carrier proteins is attributed to alterations in membrane fluidity.

ACKNOWLEDGEMENTS

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