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Cholesterol modulates organic cation transport activity and lipid fluidity in rat renal brush-border membranes

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Abstract

The role of cholesterol in organic cation transport was studied in rat renal brush-border membranes. H^+ gradient-dependent uptake of the organic cation tetraethylammonium in brush-border membrane vesicles was stimulated by cholesterol enrichment in a dose-dependent manner. The dissipation rate of the H^+ gradient, a driving force for organic cation transport in brush-border membranes, was reduced by cholesterol enrichment. Tetraethylammonium uptake in the absence of H^+ gradient was also stimulated by cholesterol enrichment. These findings indicate that cholesterol modulates tetraethylammonium uptake by affecting the intrinsic activity of the organic cation transporter and the H^+ gradient dissipation rate. Therefore, cholesterol content should be an important determinant for organic cation transport in renal brush-border membranes.

Keywords: Renal secretion; Organic cation; Tetraethylammonium; Membrane fluidity; Cholesterol; Brush-border membrane

1. Introduction

Organic cations are actively secreted from blood to urine in renal proximal tubules [1]. The recent development of methods for the isolation of brush-border and basolateral membranes has provided a great deal of information regarding the transport characteristics of organic cations in renal proximal tubules. Specific transport of organic cations such as tetraethylammonium and N^1-methylnicotinamide has been demonstrated in both brush-border and basolateral membranes [2,3]. H^+ gradient-dependent uphill transport of organic cations (H^+/organic cation antiport system) was demonstrated in brush-border membrane vesicles from the kidneys of rats, rabbits, dogs and humans [2,3]. Since the luminal pH is more acidic than the intracellular pH in proximal tubules [4], it is reasonable to assume that the inward H^+ gradient (from lumen to cell) can serve as a driving force for the secretion of organic cations. On the other hand, the organic cation transport system in basolateral membranes is insensitive to the H^+ gradient but is stimulated by an intracellular negative membrane potential and/or organic cation/organic cation exchange [2,3].

Lipid compositions and physical properties are different between brush-border and basolateral membranes of the kidneys [5–7]. For example, cholesterol and sphingomyelin contents are high in brush-border membranes, and phosphatidylcholine and phosphatidylinositol contents are high in basolateral membranes [5]. Such differences in the lipid composition of brush-border and basolateral membranes may play major roles in vectorial transport of nutrients [8]. To date, however, there have been no reports regarding the relationship between organic cation transport activity and the lipid composition of brush-border membranes.

Cholesterol is a major constituent of renal brush-border membranes and it affects many enzymes and transporter activities [8]. In the present study, we examined the role of cholesterol in tetraethylammonium transport in renal brush-border membranes by modification of cholesterol content using cholesteryl hemisuccinate (CHS), a hydrophilic cholesterol ester.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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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 [9]. The isolated membrane vesicles were suspended in a buffer comprised of 100 mM mannitol and 10 mM Hepes (pH 7.5). The membrane vesicles were used for transport and fluorescence polarization studies on the day on which they were prepared.

2.2. Modulation of cholesterol content in brush-border membranes

In vitro increases in cholesterol content in brush-border membranes were achieved by incorporation of cholesteryl hemisuccinate (CHS), a hydrophilic cholesterol ester, by a slight modification of previously described methods [10–12]. Briefly, freshly isolated brush-border membrane vesicles were suspended in 3.5% polyvinylpyrrolidone, 1.0% bovine serum albumin in phosphate-buffered saline (PBS). CHS dissolved in ethanol or ethanol alone was then added, followed by incubation in a shaking water bath at 37°C for 60min. After treatment, the membrane vesicles were washed twice in an ice-cold experimental buffer comprised of either 100 mM mannitol and 10 mM Mes (pH 6.0) or 100 mM mannitol and 10 mM Hepes (pH 7.5).

2.3. Transport studies

Uptake of labeled tetraethylammonium by brush-border membrane vesicles was measured by a rapid filtration technique. The reaction was rapidly initiated by the addition of a buffer (20 or 80 μl) containing the labeled substrate to 20 μl of membrane suspension at 25°C or 37°C. At the stated times, incubation was stopped by diluting the reaction mixture with 1 ml of ice-cold stop solution (150 mM KCl, 20 mM Hepes-Tris, pH 7.5 and 0.1 mM $HgCl_2$). The contents of the tube were immediately poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter), which were then washed once with 5 ml of ice-cold stop solution. The radioactivity of labeled substrate on the filter was determined by liquid scintillation counting.

2.4. Fluorescence polarization studies

The fluidity of brush-border membranes was assessed by measuring the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), a hydrophobic probe [13]. DPH was dissolved in tetrahydrofuran at a concentration of 2 mM and diluted 2000-fold into PBS. Brush-border membrane vesicles (300 μg protein) in PBS were incubated for 30 min at 37°C with DPH (final concentration, 1 μM). The DPH was excited at 360 nm and emission was observed at 430 nm at 25°C or 37°C using a Shimadzu spectrofluorophotometer RF-5000 (Kyoto, Japan) equipped with a polarizer attachment. The fluorescence anisotropy was calculated using the equation $r = (I_{\parallel} - I_{\perp}/I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed with the polarized light emitted parallel and perpendicular, respectively, to the excitation polarizer.

2.5. Analytical methods

Protein was determined by the method of Bradford [14], using a Bio-Rad Protein Assay Kit with bovine γ-globulin as a standard. The dissipation rate of H^+ gradient across brush-border membranes was measured by monitoring the changes in fluorescence of Acridine orange with time [15]. Briefly, 50 μl aliquots of brush-border membrane vesicles (6 mg protein per ml) were diluted into 3 ml of buffer containing 6 μM Acridine orange. The fluorescence was recorded continuously at 25°C with a Shimadzu spectrofluorophotometer RF-5000 (excitation, 493 nm; emission, 530 nm). Cholesterol content in brush-border membranes was measured by the cholesterol esterase/cholesterol oxidase method using a commercial kit (Wako Pure Chemicals, Osaka, Japan).

2.6. Materials

[1-^{14}C]Tetraethylammonium bromide (0.19 GBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA, USA). DPH was obtained from Molecular Probes (Eugene, OR, USA), Acridine orange was from Merck (Darmstadt, Germany), FCCP from Fluka (Buchs, Switzerland) and cholesteryl hemisuccinate and bovine serum albumin were from Sigma (St.Louis, MO, USA). All other chemicals used were of the highest purity available.

2.7. Statistical analysis

Data were analyzed statistically using one-way analysis of variance followed by Dunnett's *t*-test or Student's *t*-test.

3. Results

3.1. Effect of cholesterol enrichment on tetraethylammonium uptake in the presence of H^+ gradient

Cholesterol content was increased by incubation of brush-border membrane vesicles with CHS in a dose-dependent manner (Fig. 1). Accompanying this change, brush-border membrane fluidity was decreased both at 25°C and 37°C (Fig. 1).

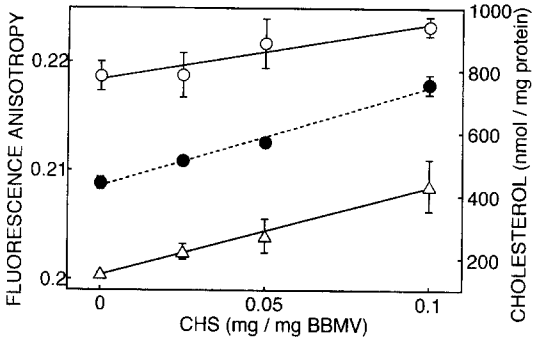

Fig. 1. Effects of CHS treatment on cholesterol content and lipid fluidity in brush-border membranes. Membrane vesicles (3 ml, 5 mg protein) were treated with various concentrations of CHS (0–0.1 mg CHS/mg protein). Fluorescence anisotropy was measured as described in Section 2 at 25°C (○) or 37°C (△). Each point represents the mean ± S.E. of six determinations. Cholesterol content (●) was measured enzymatically. Each point represents the mean ± S.E. of six determinations.

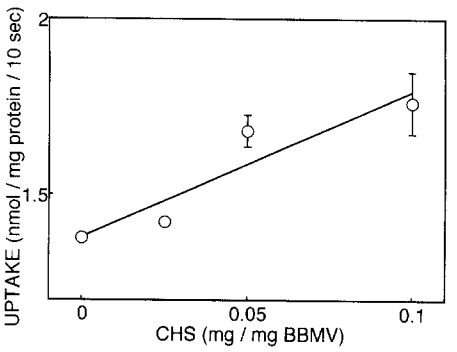

Fig. 3. Concentration dependency of the stimulation of tetraethylammonium uptake by cholesterol enrichment in brush-border membrane vesicles (with H^+ gradient). Membrane vesicles were treated with various concentrations of CHS. The uptake of [^{14}C]tetraethylammonium for 10 s at 25°C was measured as described in Fig. 2. Uptake data are expressed as the differences in tetraethylammonium uptake measured in the absence and presence of 0.1 mM cimetidine. Each point represents the mean ± S.E. of three experiments performed in 2 or 3 determinations.

Fig. 2 shows the time course of tetraethylammonium uptake by brush-border membrane vesicles in the presence of an outward H^+ gradient ($[pH]_{in} = 6.0$, $[pH]_{out} = 7.5$) at 25°C and 37°C. Under control conditions, tetraethylammonium uptake was actively driven by the outward H^+ gradient, and showed marked overshoot. The initial rate and magnitude of the overshoot of tetraethylammonium uptake were stimulated to approx. 130% of control value by cholesterol enrichment both at 25°C and 37°C. Equilibrium values of tetraethylammonium uptake in control and CHS treated vesicles were similar, suggesting that the intravesicular volume and/or the integrity of membrane vesicles was not changed by cholesterol enrichment. Nonspecific uptake of tetraethylammonium at 25°C was measured in the presence of cimetidine, which has high affinity for the H^+/organic cation antiporter [2], under the same experimental conditions as in Fig. 2A. Nonspecific uptake of tetraethylammonium were very low in both control and CHS-treated vesicles (control, 0.18 ± 0.01; CHS, 0.23 ± 0.01 nmol/mg protein/10 s, mean ± S.E. of three experiments). Therefore, changes in membrane permeability for tetraethylammonium, if any, by cholesterol enrichment were not considered further.

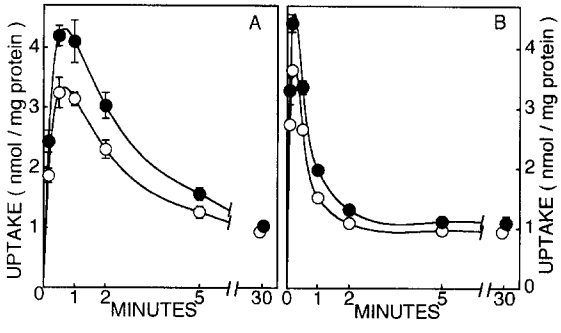

Fig. 2. Effect of cholesterol enrichment on tetraethylammonium uptake by brush-border membrane vesicles (with H^+ gradient). Membrane vesicles were treated with ethanol (0.83%) (○) or 0.05 mg CHS/mg protein (●) then the vesicles were suspended in 100 mM mannitol, 10 mM Mes (pH 6.0) and 100 mM KCl. Aliquots (20 μl) were incubated with substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM Hepes (pH 7.5), 100 mM KCl and 0.3125 mM [^{14}C]tetraethylammonium (final concentration, 0.25 mM) at 25°C (A) or 37°C (B). Each point represents the mean ± S.E. of three experiments performed in duplicate (A) or six determinations from two experiments (B).

To examine the concentration dependency of the stimulatory effect of cholesterol enrichment, the initial rate of tetraethylammonium uptake driven by an outward H^+ gradient was measured after treatment of brush-border membrane vesicles with various concentrations of CHS. H^+ gradient-dependent uptake of tetraethylammonium was stimulated by cholesterol enrichment in a dose-dependent manner (Fig. 3). A good correlation was found between fluorescence anisotropy of DPH in brush-border membranes and H^+ gradient-dependent tetraethylammonium uptake ($r = 0.989$, $P < 0.05$).

3.2. *Effect of cholesterol enrichment on the dissipation rate of H^+ gradient*

The stimulatory effect of cholesterol enrichment on tetraethylammonium uptake may be due to the decreased rate of dissipation of the H^+ gradient, the driving force for the H^+/organic cation antiport system. Therefore, we next studied the effect of cholesterol enrichment on the dissipation rate of H^+ gradient across brush-border membranes using Acridine orange, which has been used as a probe to estimate the ΔpH across membranes [15,16]. When brush-border membrane vesicles (pH 6.0) were diluted in external buffer (pH 7.5), rapid quenching of fluorescence was observed (Fig. 4A). Subsequently Acridine orange fluorescence recovered gradually with time. This latter observation reflects the dissipation phase of the H^+ gradient across the brush-border membranes. As is evident from the plots of $\ln(F_\infty - F_t)$ against time (Fig. 4B), where F_∞ is

the final fluorescence level and F_t is the fluorescence at time t, the dissipation rate of H^+ gradient was significantly decreased by cholesterol enrichment in a dose-dependent manner (control, 0.033 ± 0.003; 0.025 mg CHS/mg protein, 0.027 ± 0.003; 0.05 mg CHS/mg protein, 0.021 ± 0.002 [a]; 0.1 mg CHS/mg protein, 0.016 ± 0.002 [a] s^{-1}, mean ± S.E. of six determinations from two experiments, [a] $P < 0.05$, significantly different from control using one-way analysis of variance followed by Dunnett's t-test).

3.3. Effect of FCCP on tetraethylammonium uptake by control and CHS-treated vesicles

Effect of FCCP, a protonophore, on tetraethylammonium uptake was examined in control and CHS-treated vesicles. In the presence of FCCP, which facilitates the dissipation rate of H^+ gradient across renal brush-border membranes [15], tetraethylammonium uptake was markedly reduced in both control and CHS-treated vesicles (Fig. 5). However, even in the presence of FCCP, the tetraethylammonium uptake was slightly higher in CHS-treated vesicles than control vesicles.

3.4. Effect of cholesterol enrichment on tetraethylammonium uptake in the absence of H^+ gradient

The effect of cholesterol enrichment on tetraethylammonium uptake in the absence of H^+ gradient ($[pH]_{in} = [pH]_{out} = 7.5$) was examined. Membrane vesicles were treated with or without 0.1 mg CHS/mg protein and the vesicles were suspended in 100 mM mannitol, 10 mM Hepes (pH 7.5) and 100 mM KCl. Aliquots (20 μl) were incubated for 10 s at 25°C with substrate mixture (20 μl) containing 100 mM mannitol, 10 mM Hepes (pH 7.5), 100 mM KCl and 2 mM [^{14}C]tetraethylammonium (final concentration, 1 mM). A stimulatory effect of cholesterol enrichment on the initial rate of tetraethylammonium uptake in the absence of H^+ gradient was observed (control, 0.443 ± 0.024; CHS, 0.687 ± 0.026 [a] nmol/mg protein/10 s, mean ± S.E. of three experiments, [a] $P < 0.05$, significantly different from control using Student's t-test). These findings indicate that cholesterol modulates tetraethylammonium uptake by affecting not only the H^+ gradient dissipation rate but also the intrinsic activity of the organic cation transporter.

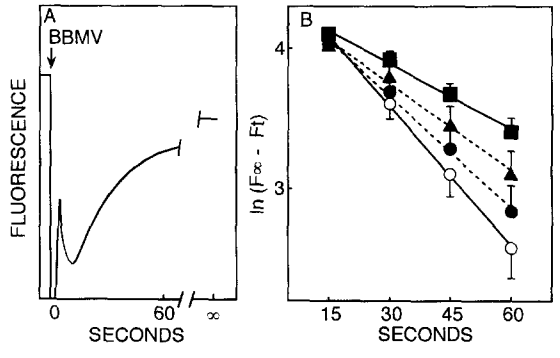

Fig. 4. Dissipation rate of the H^+ gradient in brush-border membrane vesicles. (A) Typical trace of Acridine orange fluorescence with time. External buffer contained 100 mM mannitol, 10 mM Hepes (pH 7.5), 6 μM Acridine orange and 100 mM KCl. The arrow shows the points at which brush-border membrane vesicles (50 μl), suspended in 100 mM mannitol, 10 mM Mes (pH 6.0) and 100 mM KCl, were added to 3 ml of external buffer. (B) Plots of $\ln(F_\infty - F_t)$ against time, where F_∞ is the final fluorescence level (10 min) and F_t is the fluorescence at time t, showing that the dissipation of the H^+ gradient follows first-order reaction kinetics. Membrane vesicles were treated with various concentrations of CHS: control (○), 0.025 mg CHS/mg protein (●), 0.05 mg CHS/mg protein (▲), 0.1 mg CHS/mg protein (■). Each point represents the mean ± S.E. of six determinations from two experiments.

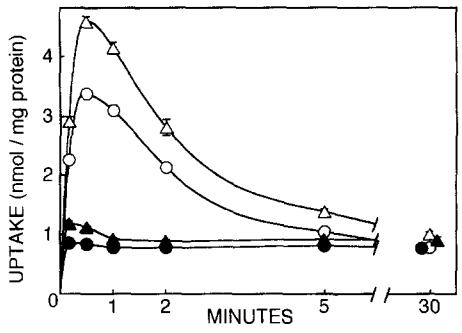

Fig. 5. Effect of FCCP on tetraethylammonium uptake by brush-border membrane vesicles (with H^+ gradient). Membrane vesicles were treated with ethanol (0.83%) (○, ●) or 0.05 mg CHS/mg protein (△, ▲), then the vesicles were suspended in 100 mM mannitol, 10 mM Mes (pH 6.0) and 100 mM KCl. Aliquots (20 μl) were incubated with substrate mixture (80 μl) comprised of 100 mM mannitol, 10 mM Hepes (pH 7.5), 100 mM KCl and 0.3125 mM [^{14}C]tetraethylammonium (final concentration, 0.25 mM) either without (○, △) or with (●, ▲) 40 μM FCCP. Each point represents the mean ± S.E. of three determinations.

4. Discussion

Cholesterol is a major constituent of plasma membrane lipids and it modulates many, but not all, membrane protein activities [5–8,10–12]. Various experimental methods have been employed to study the role of cholesterol, such as in vitro modification of cholesterol content [10–12], utilization of pathophysiological changes in cholesterol content [17,18] and use of reconstitution techniques [19]. In this study, to clarify whether renal organic cation transport is modulated by cholesterol, the effects of changes in membrane cholesterol content on tetraethylammonium uptake were examined in brush-border membrane vesicles.

It is interesting that cholesterol enrichment showed a stimulatory effect on organic cation transport (Figs. 2 and 3). One possible explanation for the stimulation of tetraethylammonium uptake by cholesterol enrichment is the changes in the dissipation rate of the H^+ gradient, the driving force for the H^+/organic cation antiport system. Our results showed that the H^+ gradient dissipation rate

was significantly decreased by cholesterol enrichment (Fig. 4). On the other hand, tetraethylammonium uptake was higher in CHS-treated vesicles than in control vesicles even in the presence of FCCP, a protonophore (Fig. 5). In addition, cholesterol enrichment also had a stimulatory effect on tetraethylammonium uptake in the absence of a H^+ gradient. Taken together, these findings suggest that cholesterol modulates organic cation transport, partly by decreasing the dissipation of the H^+ gradient and partly by increasing the activity of the transporter.

Similarly to our results, stimulatory effects of cholesterol enrichment on Na^+-dependent glucose transport were reported previously [17,18]. However, in contrast to the results regarding organic cation transport, stimulation of glucose transport was not due to the decreased rate of dissipation of Na^+ gradient, the driving force for the Na^+/glucose cotransport [17,18]. Na^+ ion permeability across brush-border membranes is not influenced by changes in cholesterol content [17,18]. This discrepancy in the changes in dissipation rate of driving forces might be due to the differences in the ion species examined.

The present results indicate that cholesterol modulates the activity of organic cation transporter in renal brush-border membranes. However, it is not known at this time whether cholesterol achieves this effect directly by acting on the transporter or indirectly through changes in membrane fluidity of the lipid bilayers. Further studies are required to clarify these points.

Cholesterol content is higher in brush-border membranes than in basolateral membranes isolated from the kidneys of rats [5], and this difference is responsible for the difference in fluidity between these two types of membranes [5]. Such difference in membrane lipid composition is maintained by tight junctions, which act as a diffusion barrier of lipids and are required for the maintenance of plasma membrane polarity [20,21]. Molitoris et al. [22,23] showed that ischemic renal injury opens tight junctions of renal epithelial cells and induces loss of polarity. As a result, cholesterol content of brush-border membranes is reduced and the fluidity is increased [22,23]. Marked alterations in brush-border membrane lipids by ischemic injury lead to a decrease in Na^+-dependent glucose transport [18]. Recently, we demonstrated that the uptake of tetraethylammonium was decreased in brush-border membrane vesicles isolated from rats with ischemic renal injury [24]. Thus, this decreased transport of organic cation may be partly due to the alteration in cholesterol content of brush-border membranes.

In summary, the present results suggest that cholesterol enrichment increases organic cation transport in renal brush-border membranes. These findings represent useful information for further studies of the role of membrane lipids in the transport of ionic drugs across the renal epithelium, under either physiological or pathophysiological conditions.

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