

Effect of Steviol on *para*-Aminohippurate Transport by Isolated Perfused Rabbit Renal Proximal Tubule

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ABSTRACT

An inhibitory effect of steviol, metabolite of the natural sweetener stevioside, on transepithelial transport of *p*-aminohippurate (J_{PAH}) was observed in isolated S_2 segments of rabbit renal proximal tubules using in vitro microperfusion. Addition of steviol (0.01–0.25 mM) to the bathing medium significantly depressed J_{PAH} (≈ 50 –90%). This inhibitory effect was dose-dependent and was maximum at a concentration of 0.05 mM. To further examine this effect, a steviol concentration (0.01 mM) that produced approximately 50% inhibition of J_{PAH} was chosen. Addition of 0.01 mM steviol to the bathing medium significantly depressed J_{PAH} by about 50 to 60%. Steviol at the same concentration (0.01 mM), when present in the tubule lumen, had no significant effect on J_{PAH} . Addition of 0.01 mM steviol to lumen and bath simultaneously, produced a slightly greater

inhibitory effect compared with addition to bath alone (60 versus 70%). A higher concentration of steviol, 0.05 mM (which maximally inhibited J_{PAH} when on the basolateral side), was required on the luminal side than on the basolateral side before an inhibitory effect was observed. To further examine the mechanism by which steviol inhibited J_{PAH} , its effect on Na^+ - K^+ ATPase activity and ATP content was determined. Steviol at concentrations of 0.01 and 0.05 mM had no effect on Na^+ - K^+ ATPase activity or cell ATP content. Kinetic analyses indicated that steviol can competitively inhibit PAH transport at the basolateral membrane. The present study clearly showed that steviol can have a direct inhibitory effect on renal tubular transport by competitive binding with organic anion transporter.

Steviol, the aglycone part of stevioside and many other natural glycosides, is one of the major metabolites of stevioside during its enzymatic hydrolysis (Hutapae et al., 1997) (Fig. 1). Stevioside is the major sweet component isolated from the leaves of *Stevia rebaudiana*. It is a complex of three glucose molecules and one molecule of steviol aglycone, a diterpenic carboxylic alcohol (Wood et al., 1995). Due to its sweetness (about 300 times sweeter than sucrose; Crammer and Ikan, 1986) and its noncaloric value, it has become popular as a sugar substitute in a variety of foods and beverages in Japan, Brazil, South Korea, and Paraguay (Fujita and Edahiro, 1979; Kinghorn and Soejarto, 1991). In addition to its use as a sweetener, several researchers have shown stevioside to have therapeutic value as a contraceptive (Planas and Kuc, 1968), and to have cardiovascular (Melis and Sainati, 1991; Melis, 1992; Chan et al., 1998) and metabolic effects (Curi et al., 1986), as well as effects on glucose absorption (Toskulkao et al., 1994). Its influence on renal function has also been suggested (Melis, 1992). Changes in renal

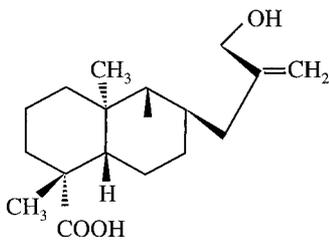
function, renal blood flow, natriuresis, and diuresis have been observed after intravenous stevioside administration.

The incubation of stevioside with intestinal bacterial microflora obtained from rats in vitro showed the complete conversion of stevioside to steviol within an incubation period of 2 to 4 days (Hutapae et al., 1997). Steviol administered either intracecally or orally was nearly completely absorbed in the lower bowel of the rat (Wingard et al., 1980). Steviol has been reported to be a toxic substance with mutagenic and bactericidal activities in *Salmonella typhimurium* TM 677 (Pezzuto et al., 1985). The inhibition of glucose absorption in hamster intestine was also observed after steviol treatment (Toskulkao et al., 1995). In contrast, steviol and stevioside have also been reported to have therapeutic value as diuretic drugs (Melis, 1997), and also as diabetic drugs by stimulating insulin secretion from pancreas (Jeppesen et al., 2000). However, detailed information concerning the toxicity of stevioside and steviol is required before their commercial use as a food additive or drug would be granted.

Investigations on the systemic effects of steviol have been performed. The intravenous infusion of steviol into rats has also been found to affect kidney function, and it induced diuresis and natriuresis with no significant change in glomerular filtration rate and renal plasma flow (Melis, 1997).

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ABBREVIATIONS: OAT, organic anion transporter; PAH, *p*-aminohippurate; J_{PAH} , net transepithelial transport of PAH; DMSO, dimethyl sulfoxide; Pi, inorganic phosphate; HPLC, high-performance liquid chromatography.



Steviol (C₂₀H₃₀O₃)
MW 318.44

Fig. 1. Chemical structure of steviol.

However, the experimental model used in the previous studies did not permit clear differentiation between the vascular and the renal tubular effects of steviol.

The renal proximal tubules serve an important function in the elimination of a wide range of xenobiotics via the organic anion and cation secretory systems (Pritchard and Miller, 1996). At present, four mammal organic anion transporter isoforms (OAT1, OAT2, OAT3, and OAT4) and a fish isoform (fROAT) have been cloned and identified. In addition, the renal-specific transporter and unknown putative transporter (UST1) have been sequenced and are candidates for the OAT family (Sekine et al., 2000). Among these transporters in the renal proximal tubule, the PAH transporter (OAT1) is regarded as the major organic anion transporter that contributes to elimination of xenobiotics with diverse chemical structure. Because of the functional importance of this secretory transport systems, interfering with or inhibiting its function could lead to an accumulation of potentially toxic compounds in the body. Steviol has been shown to inhibit the accumulation of the prototypical organic anion, *p*-aminohippurate (PAH), in rat renal cortical slices (Toskulkao et al., 1994). Thus, we hypothesized that steviol would affect the transepithelial transport of PAH in isolated S₂ segments of the rabbit renal proximal tubule as well. The present study was carried out to test this hypothesis using an in vitro microperfusion technique. This in vitro procedure has proven to be a powerful tool to study tubular function. It is very sensitive and significant changes in transepithelial transport of PAH (J_{PAH}) can be detected, even though only small changes in tubular function and metabolism occur (Chatsudthipong and Dantzler, 1991). This in vitro technique has advantages for examining the direct effect of steviol on renal secretory function under well defined conditions where any systemic effect is eliminated. In the present study, we examined the effects of steviol on transepithelial transport of PAH and some of the mechanisms by which the effects might be mediated.

Materials and Methods

Animal Preparation and Tubule Dissection. Male New Zealand White rabbits weighing 1.5 to 2.0 kg were sacrificed by intravenous injection of pentobarbital sodium. The left renal artery was infused with sucrose-HEPES-buffered medium (250 mM sucrose, 10 mM HEPES, titrated to pH 7.4 with 1 M Trizma base) to remove blood from the kidney, which was then rapidly removed and cut into thin slices. The dissection of proximal tubules from the thin slices was performed under a microscope in chilled (on ice) bicarbonate-

buffered medium (110 mM NaCl, 5 mM KCl, 2 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM sodium acetate, 1.8 mM CaCl₂, 8.3 mM D-glucose, 5 mM L-alanine, 1 mM citric acid, 1.5 mM sodium lactate, 1 mM sodium maleate, 0.9 mM glycine, and 25 mM NaHCO₃, pH 7.4, osmolality 290 mOsm/kg-H₂O), without the aid of enzymatic agents (Burg et al., 1966). The entire preparation was continuously gassed with 5% CO₂ and 95% O₂ mixture for the duration of the dissection and experiment. In the experiments, only the S₂ segment of the proximal tubule was used because maximal PAH secretion is found in this segment of mammalian renal tubules (Woodhall et al., 1978).

Perfusion of Isolated Tubules. The technique for perfusing the isolated renal tubule used in our experiment was first described by Burg et al. (1966) and modified by Dantzler (1973). Briefly, the isolated tubules were transferred to a special temperature-controlled Lucite bathing chamber containing bicarbonate-buffered medium with 3 g/100 ml of neutral dextran (40,000 ± 3,000 mol. wt.) to approximate the plasma protein concentration. The bathing medium was covered with water-saturated mineral oil to prevent the evaporation of the medium. Both ends of the tubule were held in glass micropipettes, and the tubule was perfused through another micropipette within its tip centered at the tubule lumen. The perfusion rate (10–15 nl/min) was maintained by regulating perfusion pressure with a pressure gauge. The experiments were carried out at 37°C.

Transepithelial PAH Flux. For measurements of transepithelial PAH flux (J_{PAH}), [¹⁴C]PAH was added to the bathing medium at a concentration of 20 μM. This concentration of [¹⁴C]PAH does not saturate the transport of PAH (Grantham, 1982; Dantzler et al., 1995). No PAH was present in the initial perfusate. Net transepithelial secretory transport of PAH (J_{PAH} , fmol · min⁻¹ · mm⁻¹) was determined from the amount of [¹⁴C]PAH appearing in the perfusate on the collection side and expressed per unit length of perfused tubule and time, using the following relationship (Chatsudthipong and Dantzler, 1992): $J_{\text{PAH}} = (V_C C_C)/(X_b L)$, where V_C is the fluid collection rate (in nl · min⁻¹) measured directly by collection of luminal fluid, C_C is the concentration of [¹⁴C]PAH in the collected luminal fluid (in disintegrations per minute · nl⁻¹), X_b is the specific activity of [¹⁴C]PAH in the bathing medium, and L is the length of perfused tubule (in mm), measured by ocular micrometry. The activity of [¹⁴C]PAH was determined by liquid scintillation spectrometry.

Steviol Treatment. Due to the hydrophobicity of steviol, dimethyl sulfoxide (DMSO) was added to the buffered medium to dissolve this compound. DMSO has been known to have some toxic effect on cellular function, therefore, we first tested its effect on PAH transport compared with the control value. Subsequently, we examined the concentration-response effect of steviol on J_{PAH} to select the concentration that showed 50% inhibition of control J_{PAH} to use for further study. We examined the effect of steviol on J_{PAH} when it was present 1) in the bathing medium, 2) in the perfusate, and 3) in both bathing medium and perfusate at the same time, as the presence of the drug in in vivo condition.

To examine the effect of steviol treatment on the transepithelial flux of PAH, collections were made under the following conditions: 1) with standard control solutions in both perfusate and bathing medium, with [¹⁴C]PAH in the bath before any steviol treatment (control); 2) in the presence of steviol; and 3) after the removal of steviol. During the control period, collections were made every 5 min for 15 min. At the end of these three collections, the perfusate or bathing medium or both were changed to medium containing steviol. A 5-min equilibration period was allowed after each change of solution and four 5-min collections were performed during this treatment period. Following these collections, the perfusate or bathing medium or both were changed back to the control solution, and another four samples were collected during this recovery period.

Determination of Rate of PAH Uptake across Basolateral Membrane of Intact Nonperfused Tubules. Uptake experiments were performed in a manner similar to that used previously (Chatsudthipong and Dantzler, 1991, 1992). Briefly, isolated tubules were first preincubated in bicarbonate-buffer solution at 37°C for 15 min.

The tubules were then transferred to the incubation medium containing [^{14}C]PAH (25 μM) in the presence and absence of steviol for measurement of PAH uptake. Uptake of [^{14}C]PAH was stopped after 5 min of incubation by transferring the tubules into 10 μl of 3% trichloroacetic acid, and the concentration of [^{14}C]PAH in the cell water was determined by counting in a liquid scintillation system.

Isolation of Tubule Suspensions and Treatment with Steviol. Suspensions of rabbit renal proximal tubules were isolated from New Zealand White rabbits based on the method of Vinay et al. (1981) and modified by Groves et al. (1994). The method involved digestion of renal cortex by collagenase and subsequent differential centrifugation in 40% Percoll. The final pellets were resuspended in bicarbonate-buffered solution to provide a protein concentrations of 1 and 3 mg/ml for the experiments of $\text{Na}^+\text{-K}^+$ ATPase and cell ATP content determination, respectively. Protein was measured by a modified Lowry method using Folin phenol reagent (Lowry et al., 1951). The viability of the proximal tubules was tested by using 1% trypan blue.

The renal proximal tubule suspensions were incubated with 0.01, 0.05, and 2.0 mM steviol in bicarbonate-buffered medium at 37°C, and continuously gassed with 95% O_2 and 5% CO_2 for 20 min in a shaking water bath. The tubule suspensions that were incubated with bicarbonate-buffered medium containing various concentrations of DMSO (in the absence of steviol) were used as paired controls.

Determination of $\text{Na}^+\text{-K}^+$ ATPase Activity. After the incubation period (with or without steviol), the incubated tubule suspensions from each treatment were homogenized, and the homogenate was used to determine $\text{Na}^+\text{-K}^+$ ATPase activity from the difference of amount of inorganic phosphate (Pi) liberated by the hydrolysis of ATP in the absence and presence of 1 mM ouabain. The Pi was measured by a modification of the method of Fiske and Subbarow (1925). Enzyme activity was expressed as micromoles of Pi per hour per milligram of protein.

Determination of Intracellular ATP Content. After the incubation period, the tubule suspensions for each treatment were separated and extracted to obtain total and extracellular ATP and other nucleotide contents based on the method of Mandel et al. (1990).

The separation of ATP and other nucleotides was performed by reversed phase HPLC on a 5- μm Delta Pak C_{18} column (3.9 \times 150 mm; Waters, Milford, MA) protected by a guard column using isocratic elution based on the method of Hull-Ryde et al. (1983). The mobile phase consisted of 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$, adjusted to pH 5.5 with 3 N ammonium hydroxide. The injection volume was 10 μl , and the flow rate was set at 1.2 ml/min. The values of cell ATP, ADP, AMP, hypoxanthine, and xanthine content were expressed as nanomoles per milligram of tubule protein. The separation system consisted of a model 600 pump from Waters (Milford, MA), equipped with an injection valve connected to a 20- μl sample loading loop. Peak monitoring was performed with a model 996 (Waters) photodiode array detector, which monitored the absorbance of the eluents at 254 nm using Millennium³² software. Various concentrations of ATP, ADP, AMP, hypoxanthine, and xanthine mixture (0.05, 0.025, 0.01, 0.005, and 0.002 mM) were used to construct standard curves.

Kinetic Study of Steviol Inhibition of PAH Uptake into Renal Proximal Tubule Suspensions. Initially, the time course of PAH uptake by rabbit renal proximal tubule suspensions was examined. The [^{14}C]PAH concentration used in this experiment was 5 μM , which was far below the level that would saturate the transport systems. The uptake time was varied to be 15 s, 30 s, 45 s, 1 min, 2 min, and 5 min. We then chose to perform the kinetic uptake using a 30-s uptake period that is in the linear phase. This permitted us to work in the initial phase of PAH uptake and provided ample time to get an excellent measure of radioactivity. This experiment was performed using renal proximal tubule suspensions (~0.05 g of wet weight/ml of bicarbonate-buffered medium) prewarmed at 37°C for 15 min before the uptake study so that the tubule would be fully functional at the time of study. A bicarbonate-buffered medium

containing various concentrations of PAH in the absence and presence of 0.01 mM steviol was added to the incubated tubule suspensions to obtain a final concentration of PAH at 5, 20, 100, 200, 1000, and 2000 μM . After the addition of [^{14}C]PAH, the tubule suspension was incubated at 37°C for 30 s. The uptake time was terminated by the addition of 5 ml of a cold sucrose-HEPES-buffered solution containing 1 mM probenecid, and then rapidly filtered through a glass microfiber filter (GF/G; Whatman, Maidstone, UK) with a vacuum suction, and rinsed with a 5 ml of ice-cold solution containing 1 mM probenecid. The tubules were dissolved in 0.5 ml of 1 N NaOH for at least 3 h and neutralized with 0.5 ml of 1 N HCl. The extract was counted by a liquid scintillation counter. The radioactivity was calculated for the amount of PAH uptake and expressed as picomoles of PAH uptake per milligram of tubule protein. These data were plotted as a Lineweaver-Burk plot ($1/[\text{PAH}]$ versus $1/\text{PAH uptake}$) and the K_m (Michaelis-Menten constant) was estimated from the x -axis intercept. The maximal rate of PAH transport (J_{max}) by proximal tubules was estimated from the y -axis intercept. In addition, the K_i of steviol for PAH uptake was calculated to evaluate the specificity of steviol for the PAH transporter. The K_m and J_{max} in the presence of steviol indicates whether steviol interacts with the PAH transporter. They also used to estimate the type of inhibition.

Chemicals. [^{14}C]PAH (specific activity of 40.60 mCi/mmol) was purchased from PerkinElmer Life Science Products (Boston, MA). ATP, ADP, AMP, hypoxanthine, and xanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Steviol was kindly provided by Dr. Chaivat Toskulkaio at the Department of Physiology, Faculty of Science, Mahidol University (Bangkok, Thailand). All other chemicals were analytical grade.

Statistical Analysis. Results are expressed as mean \pm S.E. The n value is the number of experiments (one tubule from a single animal was used for each experiment). In the perfusion experiments, the mean value for the three control periods was compared with the value for each experimental period in the same tubule and the values between groups at the same time period were also compared using two-way analysis of variance with repeated measure design. The significance of the difference between these values was determined with Fisher's protected least-significant difference post hoc test. For the experiments on $\text{Na}^+\text{-K}^+$ ATPase activity, cell ATP content and the rate of PAH uptake, the difference between control and experimental means was determined by a one-way analysis of variance and a post hoc test with Student-Newman-Keuls test. The comparison between control and experimental means in the kinetic study was determined by a t test for paired observations. The values were considered to be statistically significantly different when the $p < 0.05$.

Results

Initially, the experiment was performed to be certain that DMSO (used as a solvent for solubilizing steviol in the subsequent experiments) did not disturb the normal function of the isolated perfused renal proximal tubules throughout the experimental duration. The rabbit renal proximal tubule was perfused with a control solution and 0.125% (v/v) DMSO was added to the bathing medium. The transepithelial transport of PAH (J_{PAH}) did not change significantly over the 65-min period with the mean value of 595.67 ± 22.66 fmol/min/mm. This result helped us to eliminate any effects of DMSO on J_{PAH} . This meant that any alteration in J_{PAH} observed after steviol treatment would be due to the effect of steviol itself.

Concentration-Response Effect of Various Concentrations of Steviol on J_{PAH} . The effects of steviol at various concentrations were examined when steviol was only present on the basolateral side, the rate-limiting step for the transepithelial transport of PAH (Shuprisha et al., 1999). We

perfused and bathed each tubule initially with a control bicarbonate-buffered medium containing DMSO at the same concentration as that used to dissolve steviol for 15 min. This procedure was performed to obtain the control values of J_{PAH} . After this period, the bathing medium was changed to one containing steviol and J_{PAH} was determined at 10 and 30 min after steviol treatment. As shown in Fig. 2, the presence of steviol in the bath inhibited J_{PAH} in a dose-dependent manner. The calculated IC_{50} value for the J_{PAH} was 0.01 mM for steviol. To further examine the steviol action when it was present at each side of the proximal tubule, a concentration of 0.01 mM steviol, which showed a half-inhibition of J_{PAH} from the control value, was chosen.

Effect of 0.01 mM Steviol on J_{PAH} . In these experiments, the S_2 segments of rabbit renal proximal tubules were perfused and bathed initially with a control bicarbonate-buffered medium containing 0.005% (v/v) DMSO (the same concentration as that used to dissolve 0.01 mM steviol) for the first 15 min to obtain the control values of J_{PAH} . Following this period, the perfusate was changed to one containing 0.01 mM steviol. J_{PAH} was slightly decreased, but not significantly different from control values (Fig. 3a). In contrast, the addition of 0.01 mM steviol to the bathing medium caused J_{PAH} to be significantly depressed by about 50 to 60% compared with control values. The depression of J_{PAH} remained significantly different from control values even after steviol had been removed from the bathing medium for 20 min (Fig. 3b). The depression of J_{PAH} under these conditions was significantly different from that seen when steviol was in the perfusate (Fig. 3a). When 0.01 mM steviol was simultaneously added to both the perfusate and the bathing medium (Fig. 3c), there was no significant difference in the inhibitory effect on J_{PAH} compared with that observed with steviol addition to the bathing medium alone. As observed when steviol was in the bath alone, J_{PAH} remained depressed when 0.01 mM steviol was removed from both the bathing medium and the perfusate (Fig. 3c).

Effect of Luminal Steviol at a Concentration of 0.05 mM on J_{PAH} . Following no significant effect of 0.01 mM steviol in the lumen on J_{PAH} , we decided to examine the effect of a 0.05 mM steviol addition to perfusate on J_{PAH} . As shown in the concentration-response experiments (Fig. 2), 0.05 mM was the lowest concentration of steviol in the bath

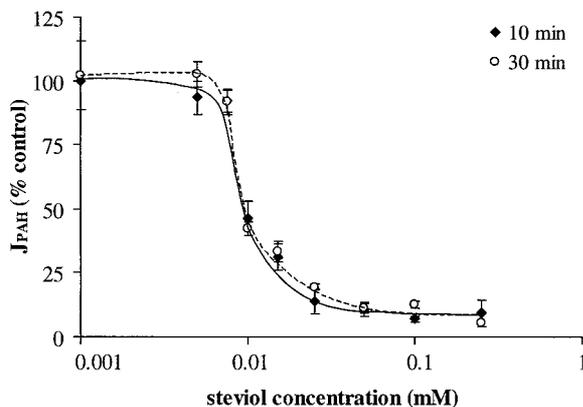


Fig. 2. Concentration-dependent inhibition of J_{PAH} in isolated perfused tubule by various concentrations of steviol in the bathing medium after incubation periods of 10 and 30 min. Each point represents mean \pm S.E. of three separate experiments.

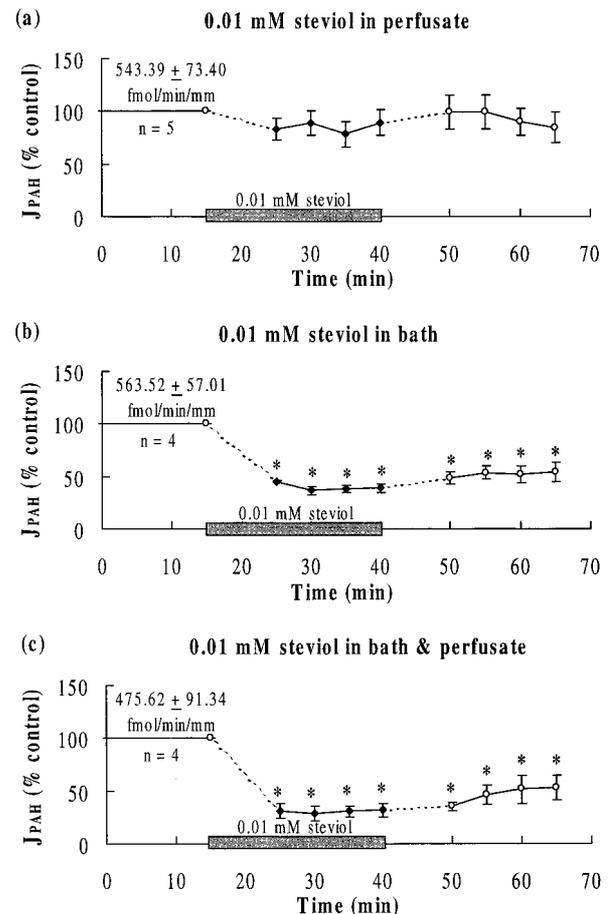


Fig. 3. Effect of 0.01 mM steviol in perfusate (a), in a bath (b), and in both a bath and perfusate (c) on J_{PAH} in tubules perfused and bathed with a bicarbonate-buffered medium containing 0.005% (v/v) DMSO. Values of J_{PAH} are shown as percentage of the mean control value (control being 100%) for each tubule in the first 15 min. Absolute mean J_{PAH} ($\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$) value \pm S.E. for all tubules in the first 15 min is indicated (n = number of tubules). The shaded bar indicates time when 0.01 mM steviol was present in the medium. *, significant difference from the control; #, significant difference from the values at the same time period of 0.01 mM steviol treatment in perfusate, $p < 0.05$.

alone at which the maximum inhibitory effect (90% inhibition) on J_{PAH} was reached. The addition of 0.05 mM steviol to the luminal side alone significantly depressed J_{PAH} from the control values by about 70% (Fig. 4). After the removal of steviol from the perfusate, J_{PAH} remained depressed for at least 20 min. This result showed that the inhibition of J_{PAH} by luminal steviol occurred but only with a higher concentration than that required on the basolateral side.

Mechanism by Which Steviol Inhibits J_{PAH} . The mechanism of transepithelial PAH transport in the proximal tubule requires energy from ATP hydrolysis and the activity of $\text{Na}^+ - \text{K}^+$ ATPase for the entry step of PAH transport across the basolateral membrane (Pritchard and Miller, 1996). As previously mentioned, transepithelial transport of PAH was inhibited by steviol (0.01 mM and higher). Thus, investigations of the effect of steviol on $\text{Na}^+ - \text{K}^+$ ATPase activity and ATP content were performed to explore the mechanism underlying the depression of J_{PAH} .

Effect of Steviol on $\text{Na}^+ - \text{K}^+$ ATPase Activity. These experiments were performed using rabbit renal proximal tubule suspensions so that enough tissue could be obtained for

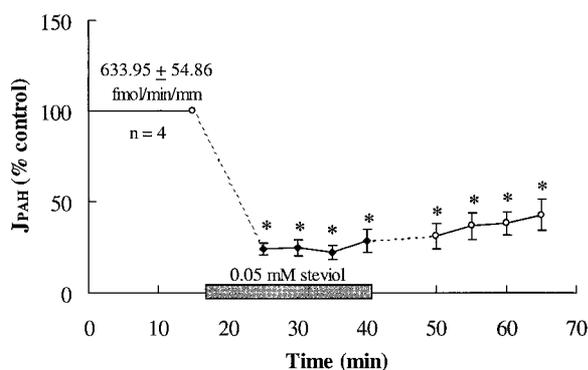


Fig. 4. Effect of 0.05 mM steviol in the perfusate on J_{PAH} in tubules perfused and bathed with a bicarbonate-buffered medium containing 0.025% (v/v) DMSO. Values for J_{PAH} are shown as percentage of mean control value (control being 100%) for each tubule in the first 15 min. Absolute mean J_{PAH} ($\text{fmol} \cdot \text{min}^{-1} \text{mm}^{-1}$) value \pm S.E. for all tubules in the first 15 min is indicated. The shaded bar indicates the time when 0.05 mM steviol was present in the perfusate. *, significant difference from control, $p < 0.05$.

the determination of $\text{Na}^+ - \text{K}^+$ ATPase activity. The proximal tubule suspension was incubated with a treatment solution (various concentrations of steviol and DMSO) for 20 min, approximately the same period used to study J_{PAH} . A bicarbonate-buffered solution was used as the control solution and various concentrations of DMSO [0.005, 0.025, and 1.0% (v/v)] were used as the paired control to match the various concentrations of steviol (0.01, 0.05 and 2.0 mM) used, respectively. As shown in Fig. 5, $\text{Na}^+ - \text{K}^+$ -ATPase activity did not change significantly after a 20-min incubation of all groups, either compared with the incubation with the control bicarbonate medium or with its paired control group for each treatment. In response to increasing the steviol concentration to 2 mM, a higher concentration than that used for the J_{PAH} studies, the $\text{Na}^+ - \text{K}^+$ ATPase activity was slightly, but not significantly, decreased from its control value. From these results, it is unlikely that the inhibitory effects of steviol observed on J_{PAH} at the concentrations used in the previous experiments involved alteration of the proximal tubular $\text{Na}^+ - \text{K}^+$ ATPase activity.

Effect of Steviol on Cell ATP Content. As previously mentioned, ATP is an important factor for the active transport of PAH across the basolateral membrane of the proximal

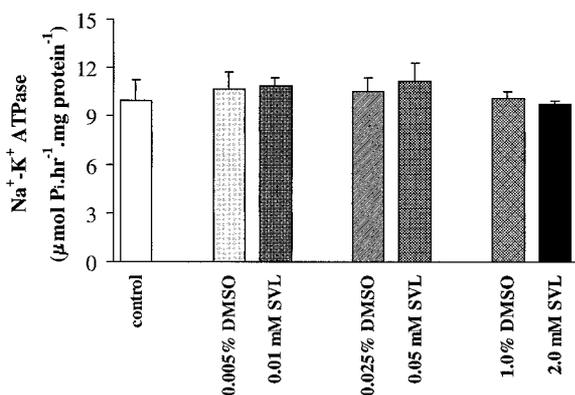


Fig. 5. Effects of various concentrations (0.01, 0.05, and 2.0 mM) of steviol (SVL) on $\text{Na}^+ - \text{K}^+$ ATPase activity in rabbit renal proximal tubule suspension after a 20-min treatment. The values are shown as micromoles of Pi per hour per milligram of tubule protein, as means \pm S.E. of duplicate measurements. $n = 4$.

tubule (Pritchard and Miller, 1996). We used reversed phase-HPLC to analyze the ATP content extracted from proximal tubules after treatment with steviol. Using HPLC analysis, low amounts of ATP and its degradation products in samples could be quite accurately detected. We found that steviol at the concentrations (0.01 and 0.05 mM) that depressed J_{PAH} in renal proximal tubules, did not significantly change the ATP, ADP, and AMP contents in the proximal tubules after a 20-min treatment (Fig. 6). In addition, the extracellular AMP and hypoxanthine contents, which are the indicators of nucleotide degradation processes, mainly appeared in the condition of cell ATP depletion, were also not significantly different from the control group in any treatment group. In contrast, steviol at a much higher concentration (2.0 mM) significantly depressed proximal tubule cell ATP content (Fig. 6a). It was found that, during the time when cell ATP content was decreased with the 2.0 mM steviol treatment, the cell AMP content was significantly increased (Fig. 6c).

Kinetic Study of Steviol on PAH Uptake in Proximal Tubule Suspensions. The protocol for this experiment was

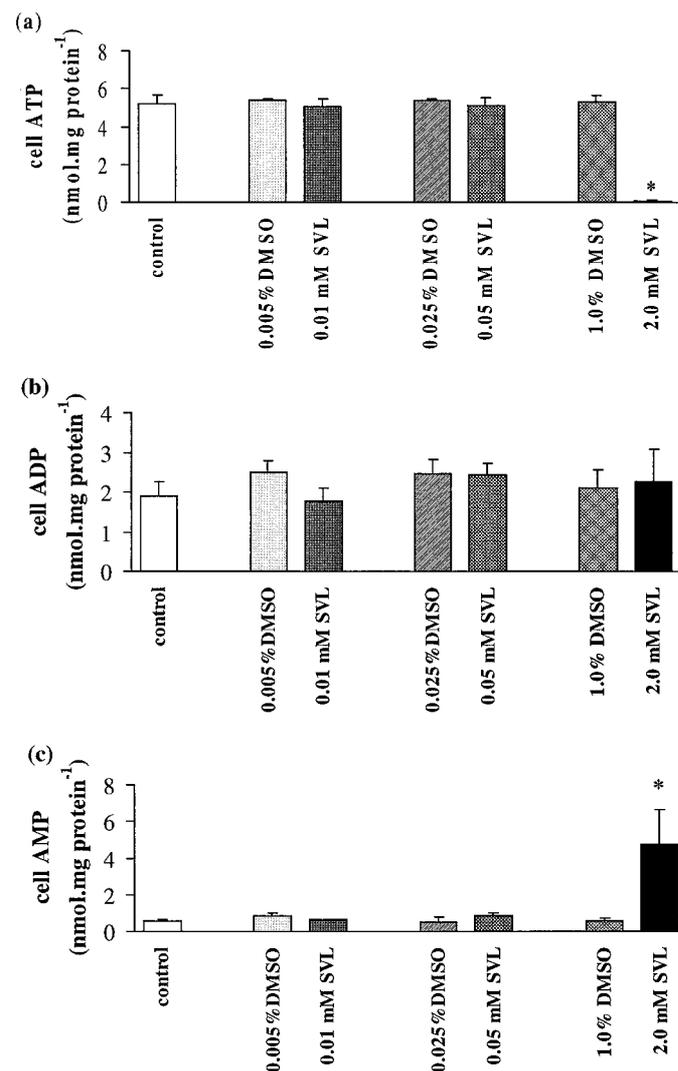


Fig. 6. Effects of various concentrations (0.01, 0.05, and 2.0 mM) of steviol (SVL) on cell ATP (a), cell ADP (b), and cell AMP contents extracted from rabbit renal proximal tubule suspensions (c) after a 20-min treatment. The values are shown as nanomoles of ATP per milligram of tubule protein; $n = 4$. *, significant difference from the other treatments, $p < 0.05$.

described under *Materials and Methods*. The experiments were performed in a paired observation between control and steviol treatment. The renal proximal tubule suspensions were incubated with medium containing various concentrations of PAH in the absence and presence of steviol for 30 s, and PAH uptake was determined and plotted using a Lineweaver-Burk plot (between $1/\text{PAH uptake}$ and $1/[\text{PAH}]$) to obtain the K_m and J_{max} of PAH uptake (Fig. 7). The K_m of PAH in the presence of 0.01 mM steviol was almost 3 times higher than the control K_m (in the absence of steviol). This showed that the affinity of the PAH transporter for PAH was reduced when the tubule was treated with steviol. The J_{max} for PAH uptake was not significantly changed by the steviol (6.0 ± 2.0 versus 6.6 ± 1.8 nmol/mg of tubule protein/min). This indicates that the inhibition of PAH transport by steviol is a competitive type. The K_i of steviol for PAH uptake in renal proximal tubule suspensions was $7.5 \pm 2.4 \mu\text{M}$. It is calculated using the equation for competitive inhibition:

$K_i \text{ value} = \text{concentration of steviol} /$

$$\left[\frac{K_m \text{ value of PAH uptake (with steviol)}}{K_m \text{ value of PAH uptake (without steviol)}} - 1 \right]$$

This shows that the affinity of the PAH transporter for steviol is high.

Effect of Steviol on Rate of [^{14}C]PAH Uptake by Isolated Nonperfused Tubules. To further examine whether the competitive binding of steviol with the PAH transport as suggested by our previous study could prevent the entry of PAH into the cells, we tested the effect of steviol on PAH uptake across the basolateral membrane of intact tubules. It was found that steviol at concentrations of 0.01 and 0.05 mM significantly depressed the rate of PAH uptake in the same manner as it depressed J_{PAH} in perfused tubules (Figs. 2 and 8). These data support the concept that the transport of PAH across the basolateral membrane is the rate-limiting step for J_{PAH} .

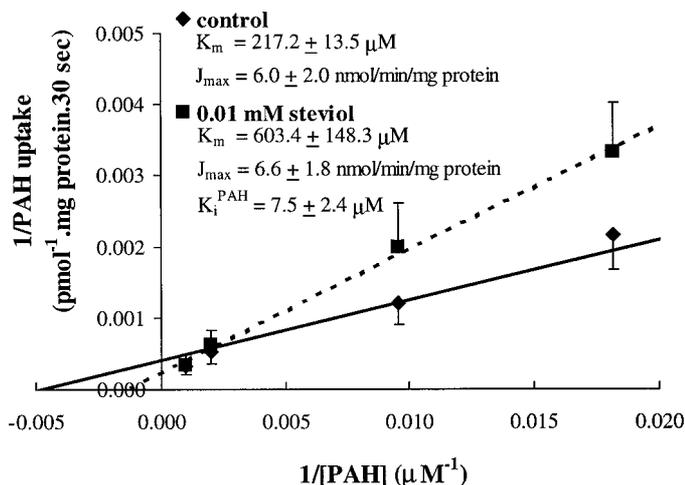


Fig. 7. Lineweaver-Burk plot of PAH uptake by rabbit renal proximal tubule suspensions with increasing concentrations of PAH in the medium in the absence (◆) and presence (■) of steviol (0.01 mM). Data are shown as the reciprocal of PAH uptake on the ordinate versus the reciprocal of PAH concentrations in the medium on the abscissa. Each point represents the mean \pm S.E. of three separate experiments.

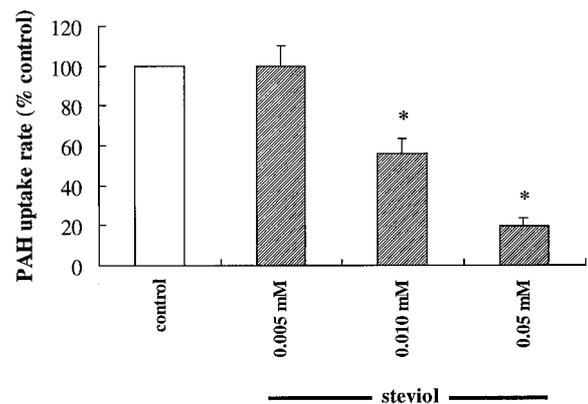


Fig. 8. Effects of various concentrations (0.005, 0.010, and 0.05 mM) of steviol in the medium on the rate of [^{14}C]PAH uptake across basolateral membrane. Tubules were preincubated with bicarbonate-buffered medium for 15 min before initiation of PAH uptake measurement. Values of PAH uptake rate from four separate experiments are shown as percent of the mean control value (control being 100%). Vertical lines are S.E. Mean control rate of uptake for all experiments was $94.75 \pm 6.29 \mu\text{mol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$. *, significantly different from control values, $p < 0.05$.

Discussion

The results of the present study with intact perfused tubules showed that steviol in the bathing medium displayed an inhibitory effect on J_{PAH} in a concentration-dependent manner (Fig. 2). The maximum inhibition was about 90% with steviol treatment at concentrations of 0.05 mM or higher. Steviol at a concentration of 0.01 mM, when it was present in the bathing medium, reduced J_{PAH} by about 50 to 60%, whereas no significant effect on J_{PAH} was observed during its presence in the perfusate. Although 0.05 mM steviol inhibited J_{PAH} when it was present in the perfusate, its effect was less marked than when it was present in the bathing medium. The presence of 0.05 mM steviol in the bathing medium depressed J_{PAH} by about 90%, but when it was present in the perfusate it depressed J_{PAH} by only approximately 70% (Figs. 2 and 4). The explanation for this observation is not known. However, it is probable that luminal steviol can pass through the luminal membrane of the proximal tubule, reaching the basolateral side and inhibiting PAH transport at the PAH transporter. The possibility that steviol, which is lipophilic, may permeate the renal cell membrane has been suggested by Yamamoto et al. (1985). The evidence that steviol can enter the proximal tubule cells, as observed in our laboratory (V. Chatsudthipong and V. Podprasart, unpublished data) from reversed phase-HPLC analysis, also supports this possibility of steviol's action, but the pathway by which steviol passed across the cell membrane is not known. Due to its long journey to the target site, some of the steviol might lose its potency. This could be why its effect on the luminal side is less than that on the basolateral side. Steviol presented on the basolateral side might get to the target site faster, leading to the higher potency observed. The most likely target site is the PAH transporter on the basolateral membrane. In this regard, it should be noted that the addition of 0.05 mM steviol to the perfusate depressed J_{PAH} to a similar degree as that observed when between 0.015 and 0.025 mM steviol was added to the bathing medium. This indicates that only half or less of the luminal steviol reached its site of action to depress J_{PAH} . Thus, with 0.01 mM in the lumen, only 0.005 mM might have reached the target

site so that no effect on J_{PAH} was observed. Unfortunately, the present study cannot confirm this possibility.

The marked depression of J_{PAH} by steviol in the bathing medium in the present study (Figs. 2 and 3) is consistent with previous studies (Toskulkao et al., 1994) in which steviol significantly inhibited PAH accumulation by rat renal cortical slices in vitro. The maximum inhibition of PAH accumulation in renal cortical slices occurred with 0.05 mM steviol treatment. The present study also found that steviol at a concentration of 0.05 mM produced the maximum inhibitory effect on J_{PAH} , although the degree of inhibition in isolated perfused proximal tubules of rabbit kidney was greater than that in rat renal cortical slices.

As mentioned earlier, transepithelial secretion of PAH by renal proximal tubules involves transport into the cell against an electrochemical gradient at the basolateral membrane, which is the rate-limiting step, followed by movement down an electrochemical gradient into the lumen across the luminal membrane. The net transport of PAH involves ATP and $\text{Na}^+\text{-K}^+$ ATPase activity. Thus, it is possible that steviol might affect these factors, leading to the reduced J_{PAH} . The possibility that steviol affects the exit step of PAH at the luminal membrane as well as uptake at the basolateral membrane also cannot be ruled out.

The exact mechanisms by which steviol exerts its effects have not been identified. We propose the four following mechanisms as possible explanations for steviol action in the reduction of J_{PAH} :

1. Steviol may act as an inhibitor of $\text{Na}^+\text{-K}^+$ ATPase activity at the basolateral membrane of the proximal tubule.
2. Steviol may enter the renal cells and then inhibit metabolic energy production within the cells.
3. Steviol may act directly on the PAH transporter at the basolateral membrane, resulting in the reduction of PAH entry into the cell, which leads to the reduction of J_{PAH} .
4. Steviol may affect the exit step of PAH at the luminal membrane.

To discriminate among these possibilities, we examined the effect of steviol on $\text{Na}^+\text{-K}^+$ ATPase activity. Steviol, at concentrations (0.01 and 0.05 mM) used to inhibit J_{PAH} in the perfusion studies, had no significant effect on this enzyme activity in rabbit renal proximal tubule suspensions (Fig. 5). The higher concentration of steviol (2.0 mM) also had no inhibitory effect on $\text{Na}^+\text{-K}^+$ ATPase activity in rabbit renal proximal tubule suspensions. In agreement with our results, steviol (2.0 mM) showed no significant change in the $\text{Na}^+\text{-K}^+$ ATPase activity of the hamster intestine, whereas it inhibited glucose absorption in the inverted gut sac and in the intestinal ring tissue of hamsters (Toskulkao et al., 1995a,b). In the present study, depression of J_{PAH} by steviol treatment at concentrations of 0.01 and 0.05 mM was observed with no alteration in $\text{Na}^+\text{-K}^+$ ATPase activity.

It is also possible that some steviol may enter the renal cell, thereby interfering with energy production and leading to the depression of tubular function. In the present study, rabbit renal proximal tubule suspensions treated with 0.01 and 0.05 mM steviol in vitro did not exhibit a reduction in intracellular ATP content, in spite of a marked inhibition of J_{PAH} in the perfused tubules. However, the cell ATP content of rabbit renal proximal tubules was markedly reduced after 20 min of 2.0 mM steviol treatment (Fig. 6). This high con-

centration of steviol was used as a positive control in the present study. This is consistent with the observation in hamster jejunum where only a high concentration of steviol (2.0 mM) inhibited ATP production (Toskulkao et al., 1995b).

Taken together, the changes in the transepithelial transport of PAH observed in this study cannot be fully explained by the alterations of $\text{Na}^+\text{-K}^+$ ATPase activity and ATP content in tubule after steviol treatment. These results indicated that steviol reduced J_{PAH} by other mechanisms. A further experiment was performed to investigate whether steviol acts on the PAH transporter to inhibit transport of PAH across the basolateral membrane. The K_m and J_{max} for basolateral PAH uptake in the present study (217 μM and 6 nmol/min/mg of protein) are consistent with those obtained from previous reports (Dantzler et al., 1995; Groves et al., 1998). The present study (Fig. 7) showed that the presence of steviol in the bathing medium reduced the affinity (increased K_m) of the transporter for PAH, but had no effect on the maximal transport rate (J_{max}). Therefore, the inhibition of J_{PAH} by steviol appeared to be competitive process. The K_i value indicated the high affinity of the PAH transporter for steviol, as seen in Fig. 7. It appears most likely that steviol affected J_{PAH} by interfering with the binding of PAH to the transporter, thereby preventing its entry into the cells. This was further supported by the finding that steviol depressed the rate of PAH uptake in isolated nonperfused rabbit renal proximal tubule in the same manner as it depressed J_{PAH} in perfused tubules (Figs. 2 and 8). These data further confirm the concept that the transport of PAH across the basolateral membrane is the rate-limiting step for the transepithelial secretion of PAH.

It was not possible to examine the fourth possibility for steviol's effect on J_{PAH} because the mechanism(s) by which PAH exits the cell across the luminal membrane is not yet known (Chatsudthipong et al., 1999). This makes it difficult to examine this possibility. However, an effect on the luminal exit step cannot be excluded.

Based on the substrate specificity of the PAH binding site (Ullrich, 1997), steviol, with a hydrophobic ring and one negative charge on the carboxylic group, seems to have the appropriate molecular structure for binding to this site. However, the results of the present study do not distinguish between transport of steviol by the PAH transporter and simple binding of steviol to the transporter.

In conclusion, the current study clearly showed that steviol has a direct inhibitory effect on renal tubular transport by competitive binding with the organic anion transporter. This prevents the entry of PAH into the cell, leading to the depression of transepithelial transport of PAH.

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