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Transport of the natural sweetener, stevioside, and its aglycone, steviol, by hOAT1 (SLC22A6) and hOAT3 (SLC22A8)

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A list of nonstandard abbreviations:

PAH, *para*-aminohippurate; hOAT, human organic anion transporter; fOat1, renal organic anion

transporter from winter flounder; ES, estrone sulfate; OR-2, oocyte ringer 2; DMSO, dimethyl

sulfoxide; TEA, tetraethylammonium; TPeA, tetrapentylammonium

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Distribution, Metabolism, & Excretion

Abstract

The natural sweetening agent stevioside and its aglycone metabolite, steviol, have been shown to inhibit transepithelial transport of *para*-aminohippurate (PAH) in isolated rabbit renal proximal tubules by interfering with basolateral entry. The aim of the present study was to determine which of the cloned basolateral organic anion transporters were involved in the renal transport of stevioside and steviol. This question was addressed in *Xenopus laevis* oocytes expressing human organic anion transporter 1 (hOAT1), 3 (hOAT3) and winter flounder OAT (fOat1). The parent compound, stevioside, had no inhibitory effect on either PAH (hOAT1) or ES (hOAT3) uptake. In contrast, steviol showed significant, dose-dependent inhibition of PAH and estrone sulfate (ES) uptake in hOAT1- or hOAT3-expressing oocytes, respectively. The IC_{50} of steviol for hOAT1-mediated PAH transport was 11.1 µM, as compared to 62.6 µM for hOAT3-mediated ES uptake. The Michaelis Menten inhibition constants (K_i) for steviol transport mediated by hOAT1 and hOAT3 were 2.0 ± 0.3 and 5.4 ± 2.0 µM, respectively. *Trans*-stimulation of PAH efflux by steviol was assessed to determine if steviol itself was transported by hOAT1 or hOAT3. A low concentration of steviol, 1 µM, increased the efflux of [^{3}H]-PAH (*trans*-stimulated) via both hOAT1 and hOAT3. In addition, it was shown by electrophysiology that steviol entry induced inward current in fOat1-expressing oocytes. In conclusion, stevioside had no interaction with either hOAT1 or hOAT3; whereas, hOAT1, hOAT3 and fOat1 were all shown to be capable of steviol transport and thus, can play a role in its renal transport and excretion.

Stevioside is a non-caloric natural sweetener isolated from the leaves of *Stevia rebaudiana*. It is about 300 times sweeter than sucrose (Bridel and Lavielle, 1931). Therefore, it is a popular sugar substitute in a variety of foods and often used as a food supplement in Japan, China, South Korea and Taiwan. Stevioside can be degraded to steviol, an aglycone, by the intestinal microflora from various animal species, including man (Hutapea et al., 1997; Koyama et al., 2003b). Stevioside is a large neutral molecule with both polar and hydrophobic regions; whereas, steviol contains a hydrophobic ring and one negative charge on the carboxylic group. The chemical structures of stevioside and steviol are shown in Figure 1. In addition to its sweetening properties, stevioside has been shown to have potential therapeutic value as a contraceptive (Melis, 1999) and an anti-hypertensive agent (Chan et al., 1998; Jeppesen et al., 2002; Jeppesen et al., 2003). Continued consumption of stevioside extract for 3 months reduced blood pressure in hypertensive patients (Chan et al., 2000). Both stevioside and steviol alter glucose metabolism (Suanarunsawat and Chaiyabutr, 1997) and glucose absorption (Toskulkao et al., 1995). They have also been proposed to have potential role as anti-hyperglycemic agents by stimulating insulin secretion from pancreatic beta cells (Jeppesen et al., 2000; Jeppesen et al., 2002; Jeppesen et al., 2003). Stevioside was not effectively absorbed from everted sacs of rat intestine; whereas, steviol was rapidly absorbed (Koyama et al., 2003b). Similarly, stevioside showed very limited absorption by the Caco-2 monolayers and steviol was much better absorbed (Geuns et al., 2003). In addition, limited intestinal secretion of steviol was also observed in these studies.

The renal handling of stevioside and steviol has also been evaluated. It has been reported that stevioside clearance was higher than inulin clearance, indicating that stevioside is secreted by renal tubular epithelium (Melis, 1992). After oral administration of steviol-17-[^{14}C] into rats, its excretion was found in the feces and urine. In addition, steviol-derived radioactivity was

largely found in the urine following bile duct ligation after oral or intracecal administration, indicating that steviol was absorbed from lower bowel and excreted by both renal and biliary system (Wingard et al., 1980). However, the underlying mechanisms of stevioside and steviol excretion are still unknown. Stevioside and steviol have also been shown to induce diuresis and natriuresis without a significant change in glomerular filtration rate or renal plasma flow (Melis, 1992; Melis, 1997). Furthermore, steviol was shown to inhibit accumulation of PAH in rat renal cortical slices (Toskulkao et al., 1994). Both stevioside and steviol have shown to inhibit transepithelial transport of PAH in isolated S2 segments of rabbit renal proximal tubule by interfering with the basolateral entry step (Jutabha et al., 2000; Chatsudthipong and Jutabha, 2001). However, this experimental model did not permit the clear elucidation of the interactions of stevioside and steviol with specific basolateral organic anion transporters.

Two organic anion transporters have been cloned in man that are expressed in the basolateral membranes of the renal proximal tubule, hOAT1 and hOAT3, and both act as organic anion exchangers (Hosoyamada et al., 1999; Cha et al., 2001; Sweet et al., 2003). Thus, they mediate the energetically uphill transport of organic anions from the blood into the cells of the proximal tubule in exchange for intracellular α-ketoglutarate, and drive tubular secretion of a wide variety of endogenous and exogenous anions including nucleotide antiviral agents, non-steroidal anti-inflammatory drugs, methotrexate, and urate (Cihlar et al., 1999; Khamdang et al., 2002; Takeda et al., 2002).

In the present study, cloned hOAT1 and hOAT3 expressed in *Xenopus laevis* oocytes were used to establish whether these transporters were involved in the transport of stevioside and steviol. These data indicate that steviol, but not stevioside, is an effective inhibitor of both hOAT1 and hOAT3 transport. Furthermore, as assessed by both *trans*-stimulation and electrophysiological studies, steviol is itself a substrate for these carriers.

Materials and Methods

Chemicals. [^{3}H]-PAH (40 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]-ES (46 Ci/mmol) was purchased from New England Nuclear Corp (Boston, MA). Unlabeled PAH and ES, probenecid, furosemide, bumetanide, indomethacin, cimetidine, methotrexate and tetraethylammonium (TEA) were purchased from Sigma (St. Louis, MO). Stevioside and Steviol were kindly provided by Dr. Chaivat Toskulkao at the Department of Physiology, Faculty of Science, Mahidol University (Bangkok, Thailand). All other chemicals were analytical grade and obtained from commercial sources.

Oocyte expression. Adult female *Xenopus laevis* were purchased from Xenopus One (Ann Arbor, MI). The animals were anaesthetized with 0.3% tricaine (Sigma), decapitated, and pithed. All animal procedures were conducted according to protocols approved by the NIEHS Animal Care and Use Committee. Stage V and VI oocytes were selected and isolated by collagenase digestion as previously described (Cihlar et al., 1999). Capped cRNA of hOAT1, hOAT3 and fOat1 was synthesized from purified plasmid cDNA using mMESSAGE mMACHINE® T7 ultra kit for ultra high yields of capped RNA (Ambion, Inc., Austin, TX). Defolliculated oocytes were injected with 20 ng of cRNA for transport and electrophysiological studies.

Measurement of PAH and ES uptake in oocyte-expressing hOAT1 and hOAT3. The *cis*-inhibitory effects (*i.e.,* inhibitor and labeled-substrate were presented to the same side of the membrane or cell) of stevioside, steviol and various compounds on PAH and ES uptake mediated by hOAT1 and hOAT3 were assessed three days post-injection. Oocytes were incubated with 1 ml of Oocyte Ringer 2 (OR-2) buffer (in mM: 82.5 NaCl, 2.5 KCl, 1 Na$_2$HPO$_4$, 1 MgCl$_2$, 1 CaCl$_2$, 1 Na-pyruvate, 5 HEPES/sodium hydroxide (NaOH), pH 7.6) containing either 10 μM [^{3}H]-PAH (hOAT1) or 100 nM [^{3}H]-ES (hOAT3) in the absence or presence of

stevioside, steviol and other test compounds for 60 min at room temperature. Both stevioside

and steviol were dissolved in dimethyl sulfoxide (DMSO) and diluted with OR-2 buffer. The

final concentration of DMSO in the incubation medium was adjusted to 1% or less and control

measurements were made in the presence of equal DMSO concentrations. Uptake was

terminated by aspiration of the incubation medium and addition 1 ml of ice-cold OR-2 buffer.

Oocytes were washed four more times with ice-cold OR-2 and placed in individual scintillation

vials, lysed with 1 N NaOH and neutralized with 1 N hydrochloric acid (HCl). Four ml of

scintillation fluid (Ecolume™, ICN, Irvine, CA) was added and radioactivity was measured using

a Tri-Carb™ 2900TR Liquid Scintillation Analyzer (Packard, Meriden, CT). The uptake of PAH

and ES was expressed as a mean percentage of the control.

Kinetic analysis. Human OAT1- and hOAT3-expressing oocytes were incubated with OR-2

buffer containing either [^{3}H]-PAH (hOAT1) or [^{3}H]-ES (hOAT3) at various concentrations in the

absence or presence of 50 µM steviol for 30 min (approximates initial rate of uptake for either

substrate into oocytes) (data not shown). Data were plotted as double reciprocal plots. The

inhibitory constants (IC_{50}) were calculated from sigmoidal dose-response analysis using

GraphPad Prism version 4.00 for windows (GraphPad Software, San Diego, CA). The Michaelis

Menten constants (K_m) were calculated from the interception of x-axis; whereas, the maximum

rate of organic anions uptake (V_{max}) were estimated from y-axis intercept. The Michaelis Menten

inhibition constants (K_i) were calculated by fitting the data to the following equation for

competitive inhibition (Cheng and Prusoff, 1973).

$$K_i = \frac{IC_{50}}{\left\{ 1 + \dfrac{\text{concentration of steviol}}{K_m} \right\}}$$

Determination of PAH efflux from hOAT1- and hOAT3-expressing oocytes. Three days post-injection, oocytes were loaded with OR-2 buffer containing 10 μM [^{3}H]-PAH for 120 min to generate an intracellular accumulation of [^{3}H]-PAH. Oocytes were then rinsed with room temperature OR-2 buffer. Subsequently, oocytes were incubated with OR-2 buffer in the presence or absence of 1 mM unlabeled PAH, probenecid and various concentrations of steviol for 90 min. At the end of the efflux period, [^{3}H]-PAH in the efflux medium and intracellular accumulation were measured using liquid scintillation counter as described above. Results were expressed as a mean percentage of the total PAH present in the oocytes that appeared in the efflux medium after 90 min of efflux.

Electrophysiology. When human or flounder Oat1 were expressed in *Xenopus* oocytes, inward currents were generated upon the presentation of substrate, indicating that both isoforms exhibit electrogenic exchange of organic anion for dicarboxylate (Burckhardt et al., 2000; Aslamkhan et al., 2003a). However, the magnitudes of the currents seen were considerably greater for the flounder clone. Thus, we used fOat1-expressing oocytes to directly investigate whether steviol was itself transported by the basolateral organic anion transporters. Three to four days post-injection, fOat1-expressing oocytes were placed in the 1.0 mm well of a RC-1Z oocyte recording chamber (Warner Instruments; Hamden, CT) and continuously bathed at a rate of 4 ml/min with OR-2 buffer at room temperature. The conventional two-electrode (3 M KCl; resistance of 0.5 – 5 MΩ) voltage clamp method (Geneclamp 500B, Axon Instruments, Foster City, CA) was used for measuring oocyte membrane currents. The oocyte membrane potential was held at –80 mV, which is lower than the physiological membrane potential of the proximal tubule cell *in situ* to increase current evoked by added organic anion substrates, *e.g,* PAH or steviol. OR-2 buffer containing either PAH or steviol was applied to the oocytes in a pulsed manner via solenoid-

controlled valves. Recordings were sampled at 50 Hz with a 10 Hz filter frequency. After

sampling, current signals were filtered with a Gaussian low band pass filter (2 Hz cut-off).

Statistics. Data were expressed as means ± S.E. Statistical differences were assessed using one-

way analysis of variance with Dunnett's test. In the efflux experiment, differences in efflux were

analyzed using two-way analysis of variance with randomized-block design. Differences were

considered to be significant when *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control.

Results

***Cis*-inhibition of hOAT1- and hOAT3-mediated PAH and ES uptake.** To determine the
relative inhibitory effectiveness of various organic compounds including stevioside and steviol
on PAH and ES uptake, *cis*-inhibition studies were conducted in oocytes expressing hOAT1 and
hOAT3. All compounds were tested at 100 µM. As shown in Figure 2A, PAH uptake by hOAT1
was markedly reduced by furosemide, steviol, unlabeled PAH, probenecid and indomethacin;
whereas, several compounds including TEA, stevioside, cimetidine, methotrexate, unlabeled ES
and bumetanide did not affect hOAT1-mediated PAH uptake. The inhibitory effects of these
compounds on hOAT3-mediated ES uptake were also assessed (Figure 2B). At 100 µM,
stevioside and TEA had no inhibitory effect; whereas, unlabeled PAH, steviol, cimetidine,
methotrexate, indomethacin, unlabeled ES, bumetanide, probenecid and furosemide significantly
inhibited hOAT3-mediated ES uptake.

To compare the effectiveness of stevioside and steviol with known inhibitors of hOAT1
and hOAT3, dose response experiments were conducted. As shown in Figure 3A, 50 µM to 1
mM stevioside had no effect on PAH uptake in hOAT1-expressing oocytes. In contrast, 10 µM
to 1 mM steviol significantly inhibited hOAT1-mediated PAH uptake in a dose-dependent
manner with an IC_{50} of 11.1 µM. When the effect of stevioside on ES uptake was investigated in
oocytes expressing hOAT3, no inhibition was seen, even at 1 mM stevioside. In contrast, steviol
significantly inhibited hOAT3-mediated ES uptake in a dose dependent manner (IC_{50} 62.6 µM)
(Figure 3B).

Kinetics of steviol on PAH and ES uptake. To determine the affinity of hOAT1 and hOAT3
for steviol, kinetic analyses were performed. As shown in Figure 4A, [^{3}H]-PAH concentrations
were varied from 3 to 30 µM. The rate of PAH uptake by the hOAT1-expressing oocytes was
linear for 30 min (data not shown). Thus, this time was used to estimate the initial rate for

Lineweaver-Burk analysis of hOAT1-mediated PAH uptake to determine the kinetic parameters (K_m, V_{max} and K_i) in the presence or absence (control) of steviol (summarized in Table 1). The calculated K_m of hOAT1-mediated PAH uptake doubled in the presence of 50 μM steviol. The calculated K_i value for steviol was 2.0 ± 0.3 μM. In the hOAT3 experiments, the [3H]-ES concentration was varied from 2 μM to 9 μM (Figure 4B). The estimated K_m for ES in the presence of 50 μM of steviol was 2-fold higher than the control (Table 1), with the calculated K_i 5.4 ± 2.0 μM

Trans-stimulation of hOAT1- and hOAT3-mediated PAH and ES uptake. Although the above results demonstrated that steviol effectively inhibited both hOAT1 and 3, it remained uncertain whether steviol could be transported by these basolateral OATs. Therefore, _trans_-stimulation of the of hOAT1 and hOAT3 transport by steviol was investigated. _Trans_-stimulation takes place when the presence of one transported substrate on the opposite, or _trans_, side of the membrane increases the flux labeled substrate. T_rans_-inhibition can also occur when an agent presented on the opposite side of the membrane reduces flux of the labeled-substrate, _e.g.,_ by binding tightly to the carrier and reducing the effective number of carriers available to mediate labeled-substrate transport. As shown in Figure 5A, the efflux of [3H]-PAH significantly increased by the addition of 1 mM unlabeled PAH in the incubation medium. Similarly, 1 μM steviol significantly _trans_-stimulated [3H]-PAH efflux; whereas, at higher concentrations (10 μM – 1 mM) _trans_-stimulation was not seen. In fact, 100 μM and 1 mM steviol significantly _trans_-inhibited [3H]-PAH efflux. This result was comparable to that seen when 1 mM probenecid was applied. Human OAT3-mediated [3H]-PAH was also significantly _trans_-stimulated when 1 mM unlabeled PAH was added to the external buffer. External addition of 1 μM steviol also increased the efflux of [3H]-PAH. However, this effect was not observed at

10 and 100 μM of steviol, and at 1 mM, steviol (like probenecid) *trans*-inhibited [³H]-PAH

(Figure 5B).

Electrophysiology. Since human, rat and flounder OAT1 have all been shown to be

electrogenic, exchanging one monovalent organic anion for one divalent dicarboxylate

(Burckhardt et al., 2000; Aslamkhan et al., 2003a), it should be possible to directly assess

whether stevioside or steviol were being transported via electrophysiology. To do this, voltage

clamp experiments using fOat1-expressing oocytes were performed. As noted above, flounder

OAT1 shows a greater electrical signal than rat or human OAT1. Since the signal resulting from

transport of stevioside (if any) and steviol might be modest, flounder Oat1 was chosen for these

experiments. As shown in Figure 6A, at a holding membrane potential of –80 mV, addition of

100 μM PAH induced an inward current of -61.9 ± 2.4 nA. Addition of 100 μM steviol evoked

membrane inward current of -11.7 ± 1.0 nA, indicating that steviol is translocated by fOat1;

whereas, inward current was not observed upon addition of 100 μM of stevioside (Figure 6A).

As shown in Figure 6B, the magnitude of the inward current induced by steviol was

concentration dependent. Control water-injected oocytes did not show inward current upon

addition of PAH and steviol (data not shown). To determine if steviol translocation is in fact

slower than PAH, we assessed the effect of low (1 μM) and high (100 μM) steviol

concentrations on the PAH induced current. Co-application of 1 μM of steviol with 60 μM PAH

(K_{50} for fOat1 PAH current) (Burckhardt et al., 2000), resulted in a slightly larger current than

the application of 60 μM PAH alone (103.9 ± 1.2% of control PAH current; Figure 6C).

Moreover, co-application of 100 μM of steviol with 60 μM PAH resulted in a greatly reduced

current (47.3 ± 5.4% of control PAH current; Figure 6C), paralleling its effect in the *cis*-

inhibition experiments described above (Figure 2A; 3A). Thus, it appears that although steviol is

a substrate for fOat1, its rate of translocation is markedly less than that of PAH.

Discussion

Stevia extracts and stevioside have been widely used as a low-calorie sweetener in Japan, China, South Korea and Taiwan, and this compound was approved as a food supplement in USA (Geuns, 2003). Indeed, stevioside seems to have little or no acute toxicity (Matsui et al., 1996; Toskulkao et al., 1997). Likewise, chronic stevioside consumption is believed to pose little human risk based on findings such as those of Yamada *et al.* (Yamada et al., 1985) that showed oral consumption of stevioside in amounts as high as 550 mg/kg BW/day (i.e., more than 200 times its likely maximum intake in man of about 2 mg/kg BW/day (Xili et al., 1992)) for 2 years, had no toxic or carcinogenic effects in the rat. However, pharmacological effects are suggested by other studies. For example, stevioside administration was reported to reduce blood pressure and blood glucose levels in humans (Chan et al., 1998; Jeppesen et al., 2000; Jeppesen et al., 2002; Jeppesen et al., 2003). Furthermore, its aglycone metabolite, steviol, was reported to be mutagenic and bactericidal in *Salmonella typhimurium* TM677 (Perzzuto et al., 1985). Thus, the spectrum of its biological effects and potential for adverse interactions with other pharmacological reagents remains uncertain.

Stevioside absorption, distribution and metabolism have been evaluated in experimental animals. Absorption of stevioside by everted sacs of rat intestine and Caco-2 cell monolayers was very poor; whereas, its aglycone, steviol, was rapidly absorbed (Geuns et al., 2003; Koyama et al., 2003b). Since stevioside can be degraded to steviol by intestinal microflora from various animal species, including man (Cardoso et al., 1996; Hutapea et al., 1997; Geuns, 2003; Koyama et al., 2003a), it has been suggested that following stevioside consumption only steviol is absorbed. Furthermore, it appears that stevioside shows nearly complete metabolic conversion to steviol *in vivo*. Thus, the "acceptable daily intake" of 7.9 mg/kg BW/day stevioside suggested by Xili *et al.* (1992) would yield a maximum plasma concentration of approximately 0.2 mM

steviol, assuming that oral stevioside would be fully converted to steviol and fully absorbed by

the gut *in vivo.* A recent *in vivo* study in rats gave plasma values in the same range. Peak

plasma steviol concentration was approximately 18 μM 2 hrs after single oral administration of

stevia extract (equimolar dose of 45 mg/kg BW steviol), and 15 min after oral administration of

steviol itself (45 mg/kg BW) plasma steviol reached a peak of 57.4 μM (Koyama et al., 2003a).

Based on these estimates, we have used concentrations of 0.05 to1 mM for stevioside and 0.01 to

1 mM steviol to bracket the likely exposure experienced by man.

In addition, stevioside and its metabolites are very rapidly eliminated from the body. For

example, following intravenously injection of $[^{131}I]$-stevioside into the rat, plasma radioactivity

decreased rapidly to only 6% of injected dose in the first 60 min. The excreted label was equally

found in urine and feces (Cardoso et al., 1996). Moreover, in the rat, stevioside renal clearance

was higher than inulin clearance, suggesting active tubular secretion of stevioside (or its

aglycone) by renal tubular epithelium (Melis, 1992). Similarly, the excretion of steviol-17-$[^{14}C]$

was largely found in urine of bile duct ligated rats after both oral and intracecal administration

(Wingard et al., 1980). In keeping with the possibility of tubular transport and secretion, steviol

was shown to inhibit PAH uptake in rat renal cortical slices (Toskulkao et al., 1994).

The two basolateral organic anion/dicarboxylate exchangers, OAT1 and OAT3, drive the

critical energy-dependent uphill step in organic drug and xenobiotic secretion, and thus, control

the renal elimination of endogenous and exogenous anionic drugs and metabolites from the body

(Hosoyamada et al., 1999; Cha et al., 2001; Sweet et al., 2003). Together, the rapid renal

clearance of stevioside/steviol from the body and the ability of steviol to inhibit PAH transport

suggest that one or both agents may use these transporters as a route for the excretion into urine,

reducing their systemic concentration and potential for toxicity. However, it should be noted that

if they are substrates for these carriers, they may also demonstrate significant renal accumulation

and increased potential for proximal tubular damage. They may also alter the handling of other organic anion substrates by these systems, changing their retention and potential for adverse effects. Hence, it is essential to gain a molecular understanding of the mechanisms by which both stevioside and steviol are transported by the kidney. The recent studies of Jutabha *et al.* (Jutabha et al., 2000) suggest that inhibition of organic anion transport also occurs at the tubular level. They found that 0. 7 mM stevioside modestly reduced PAH transport; whereas, only 10 μM of steviol was needed to significantly inhibit transepithelial PAH transport by the rabbit renal proximal tubule (Chatsudthipong and Jutabha, 2001). However, those studies did not assess the interactions of stevioside and steviol with specific basolateral OATs. In the studies reported above, we have demonstrated that stevioside itself did not inhibit either hOAT1 or hOAT3 (Figures 2A,B; 3A,B). Likewise, our electrophysiological analysis showed that stevioside did not produce membrane currents in oocytes expressing fOat1 (Figure 6A), suggesting that stevioside had no interaction with either hOAT1 and hOAT3 and thus, that these OATs are unlikely to mediate basolateral uptake of stevioside. However, this finding is in contrast with previous *in vivo* studies that found urinary stevioside clearance to be greater than that of inulin (Melis, 1992; Cardoso et al., 1996). In light of the present data using the cloned transporters, two explanations for the earlier data are possible. First, it is possible that the stevioside used in the earlier studies contained other stevioside derivatives or that some stevioside metabolism occurred *in vivo* after its injection. Alternatively, another transport process may be responsible for these *in vivo* findings. For example, very recently a novel organic anion transporter from human kidney (OATP4C1) has been cloned and characterized (Mikkaichi et al., 2004). This basolateral transporter appears to handle organic anions, as well as digoxin and ouabain, large neutral cardiac glycosides. Hence, OATP4C1 may play a possible role for transport stevioside. However, this possibility needs to be explored.

In contrast, the aglycone stevioside metabolite, steviol, gave very different results. Steviol very effectively inhibited hOAT1- and hOAT3-mediated transport in a dose dependent manner (Figures 2A,B; 3A,B). The inhibition of OAT1 produced by steviol was as great as that produced by equal concentrations of unlabeled PAH and furosemide (Figure 2A), suggesting that the affinity of steviol approximates that of these compounds for hOAT1. Indeed, it was nearly as effective against hOAT1 as probenecid. Against hOAT3, it was a more effective inhibitor than PAH, but it was much less effective than estrone sulfate, bumetanide or probenecid (Figure 2B), all of which are known to have a high affinity for hOAT3 (Wright and Dantzler, 2004). However, these *cis*-inhibition experiments only indicate that steviol has a high affinity for basolateral OAT transporter binding, and leave open the question of whether or not steviol is itself translocated by the cloned OATs. To address this possibility, we took advantage of the observation that, like hOAT1 and rOat1, fOat1 shows a 1:1 coupling of organic anion and dicarboxylate and shows an inward positive current upon exposure to transported substrates (Aslamkhan et al., 2003a; Aslamkhan et al., 2004; Burckhardt et al., 2000). Moreover, that current is more robust than that seen with the mammalian isoforms, in effect amplifying the signal associated with substrate transport. Using this system, we were able to demonstrate current movements upon exposure of fOat1-expressing oocytes to steviol. As shown in Figure 6A and B, both 1 and 100 µM steviol induced an inward current in fOat1-expressing oocytes. Additionally, consistent with its transport by hOAT1 and hOAT3, we observed that 1 µM steviol *trans*-stimulated the efflux of [³H]-PAH mediated by both hOAT1 and hOAT3 (Figures 5A, B). However, at higher concentrations steviol *trans*-inhibited [³H]-PAH efflux, similar to the effect of probenecid which binds very tightly to the carrier. The reason for differences in the effects of low (1 µM) and high (100 µM) steviol concentrations on PAH efflux is not clear, but may indicate that steviol translocation has a low turnover rate. Thus, at high concentrations it may

mask the binding site, and, like probenecid, produce *trans*-inhibition. Indeed, that rate of steviol

translocation (i.e., current induction) is clearly lower than that of PAH (Figure 6A, B, C). This

finding is similar to that of Groves *et al.* who showed that the tightly bound substrate,

tetrapentylammonium (TPeA), completely blocked TEA efflux from isolated rabbit proximal

tubules. The investigators proposed that TPeA binds to the organic cation transporter and

markedly slows carrier turnover rate (Groves et al., 1994; Bednarczyk et al., 2003).

   In conclusion, natural sweetener, stevioside, is not transported via basolateral organic

anion transporters. In contrast, both hOAT1 and hOAT3 transport steviol, the aglycone

metabolite stevioside, indicating that transport of steviol by these carriers may be responsible for

the renal secretion of stevioside-derived radioactivity seen previously *in vivo*. Furthermore,

steviol has a high affinity for both hOAT1 and hOAT3 and therefore, has significant potential to

diminish the renal clearance of anionic drugs and their metabolites.

Acknowledgements

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Footnotes

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Figure Legends

Figure 1. Chemical structures of stevioside and its aglycone metabolite, steviol.

Figure 2. Inhibition of (A) hOAT1-mediated [^{3}H]-PAH uptake and (B) hOAT3-mediated [^{3}H]-ES uptake. Oocytes-expressing hOAT1 **(A)** and hOAT3 **(B)** were incubated with OR-2 buffer containing either 10 μM of [^{3}H]-PAH or 100 nM of [^{3}H]-ES in the absence (control) or presence of 100 μM test compounds for 60 min. The results are expressed as a mean percentage of control from three separate experiments $\pm$ SE. Mean control PAH and ES uptake were 22.1 ± 2.1 and 0.7 ± 0.1 pmol/oocyte/hr, respectively. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Figure 3. Concentration dependence of stevioside and steviol inhibition of hOAT1- and hOAT3-mediated uptake. Oocytes-expressing hOAT1 **(A)** and hOAT3 **(B)** were incubated for 60 min with OR-2 buffer containing either 10 μM [^{3}H]-PAH **(A)** or 100 nM of [^{3}H]-ES **(B)** in the absence (control) or presence of various concentrations of stevioside and steviol. Unlabeled PAH (1 mM) and probenecid (1 mM) were used to illustrate complete block of mediated PAH and ES uptake. Water-injected oocytes showed minimal PAH and ES uptake. The values are expressed as a mean percentage of control from three separate experiments $\pm$ SE. Mean control PAH and ES uptake were 47.5 ± 8.3 and 0.6 ± 0.2 pmol/oocyte/hr, respectively. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Figure 4. Kinetic analysis of the inhibitory effect of steviol on hOAT1- and hOAT3-mediated uptake. Oocytes-expressing hOAT1 **(A)** and hOAT3 **(B)** were incubated with OR-2 buffer containing either 3 to 30 μM [^{3}H]-PAH **(A)** or 2 to 9 μM [^{3}H]-ES **(B)** in the absence (□)

or presence of 50 µM steviol (■) for 30 min. Each point represents mean ± SE of three separate experiments.

Figure 5. Effect of steviol on [³H]-PAH efflux by hOAT1- and hOAT3-expressing oocytes. Oocytes expressing hOAT1 **(A)** and hOAT3 **(B)** were loaded with OR-2 buffer containing 10 µM of [³H]-PAH for 120 min, and then incubated with OR-2 buffer in the presence or absence (control) of 1 mM PAH, 1 mM probenecid, or 1 µM to 1 mM steviol for 90 min. The values are expressed as a mean percentage of the efflux ± SE of three separate experiments. Total accumulation averaged 403.1 ± 103.2 pmol/oocyte/hr (hOAT1) and 44.6 ± 9.5 pmol/oocyte/hr (hOAT3). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Figure 6. Electrophysiological recording of PAH, stevioside and steviol induced membrane current (I_{mem}) in fOat1-expressing oocytes. Oocytes expressing fOat1 were voltage clamped at holding potential (V_h) –80 mV, and membrane currents were recorded. **(A)** 100 µM PAH, stevioside, or steviol were applied to the oocytes in a pulse manner and continuous current was monitored. Inward currents were observed in response to PAH and steviol; whereas, stevioside-evoked current was not observed. **(B)** Effects of 1 and 100 µM steviol on the fOAT1-mediated positive inward current. **(C)** PAH (60 µM) with or without steviol (1 and 100 µM doses) was applied to the oocytes in a pulsed manner. Experiments were repeated three times in oocytes from 2-3 animals. The data shown are from a representative experiment.

Table 1. Kinetic parameters for the inhibition of hOAT1-mediated PAH transport and hOAT3-mediated ES transport by steviol in *Xenopus* oocytes. Analysis was by Lineweaver-Burke linear transformation as described in Methods. The experimental conditions were as described in the Legend of Figure 4, and the data are expressed as mean ± SE of three separate experiments. Although the apparent K_m for hOAT1 and hOAT3 were doubled by steviol, these differences were only marginally significant by unpaired t-test ($p<0.10$ for hOAT1 and $p<0.15$ for hOAT3).

Cloned	K_m (μM)		V_{max} (ρmol/oocyte/30 min)		K_i (μM)
	Control	50 μM Steviol	Control	50 μM Steviol	
hOAT1	11.0 ± 1.7	21.8 ± 4.8	53.5 ± 13.0	30.6 ± 8.5	2.0 ± 0.3
hOAT3	4.8 ± 1.9	10.1 ± 2.3	14.4 ± 4.2	15.4 ± 2.3	5.4 ± 2.0
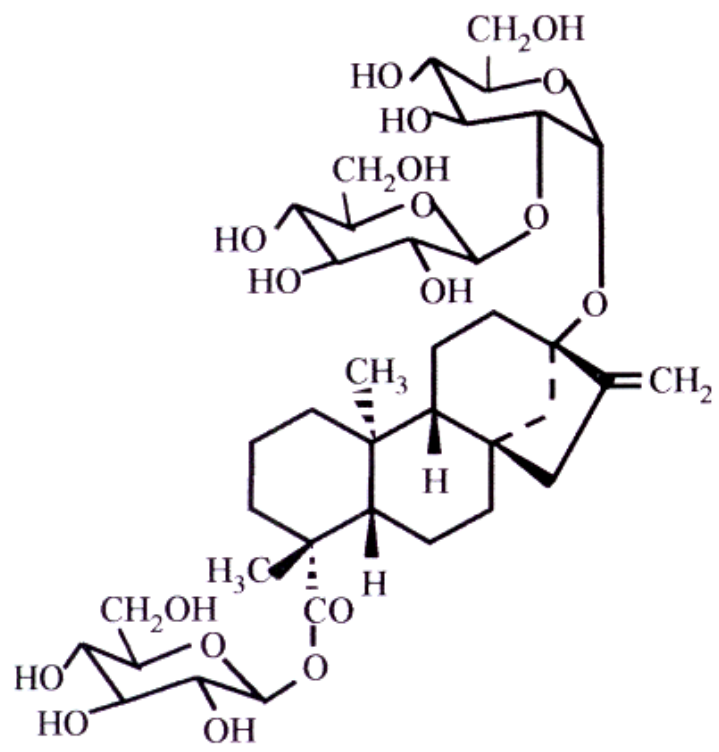
Stevioside ($C_{38}H_{60}O_{18}$)

MW 804.9
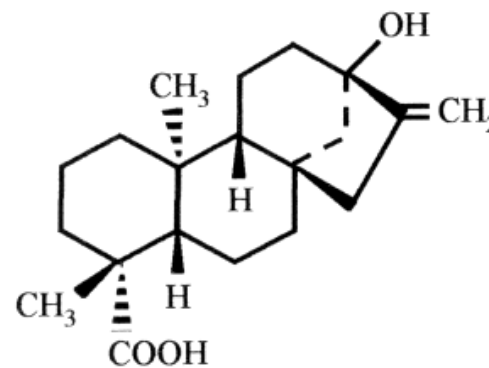
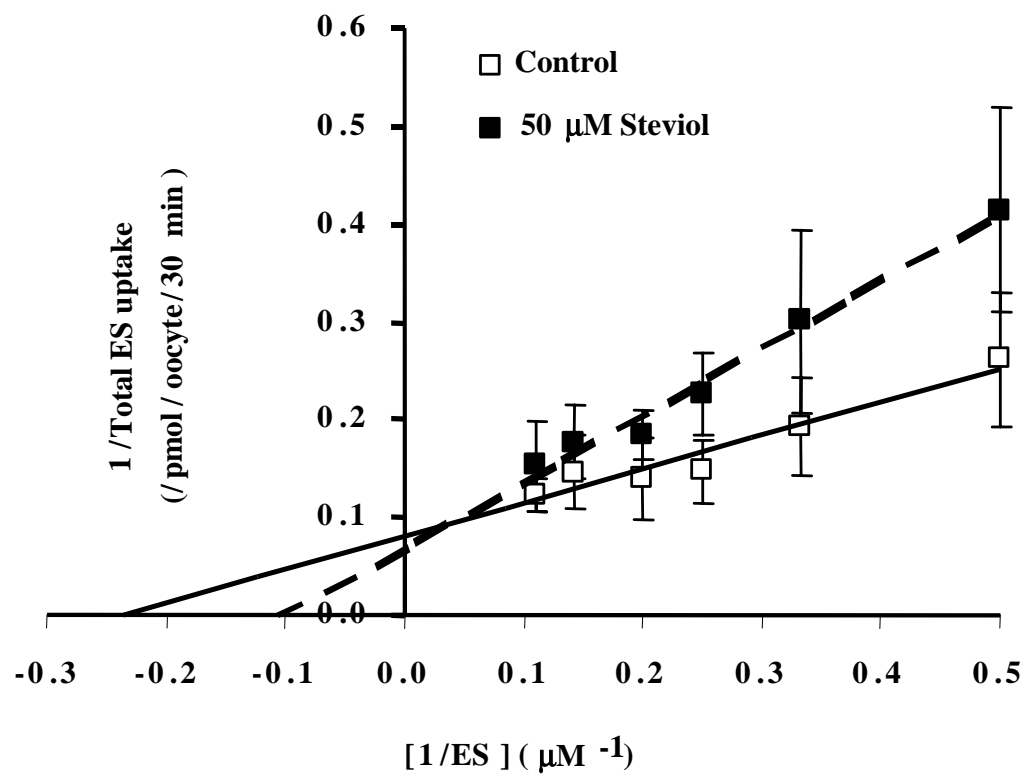Steviol ($C_{20}H_{30}O_3$)

MW 318.44
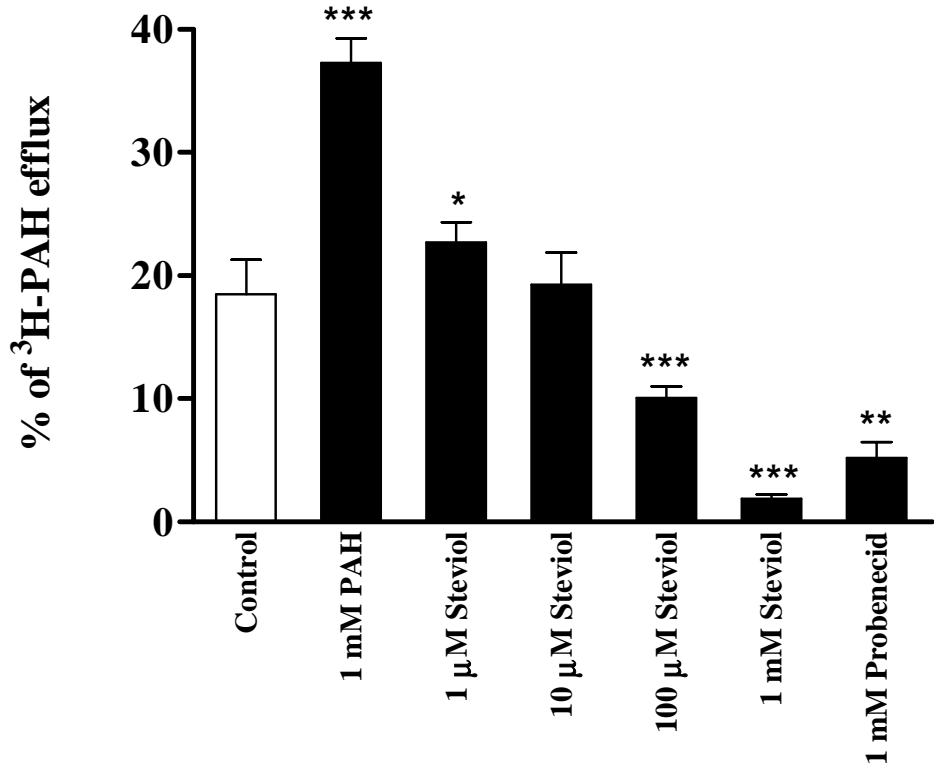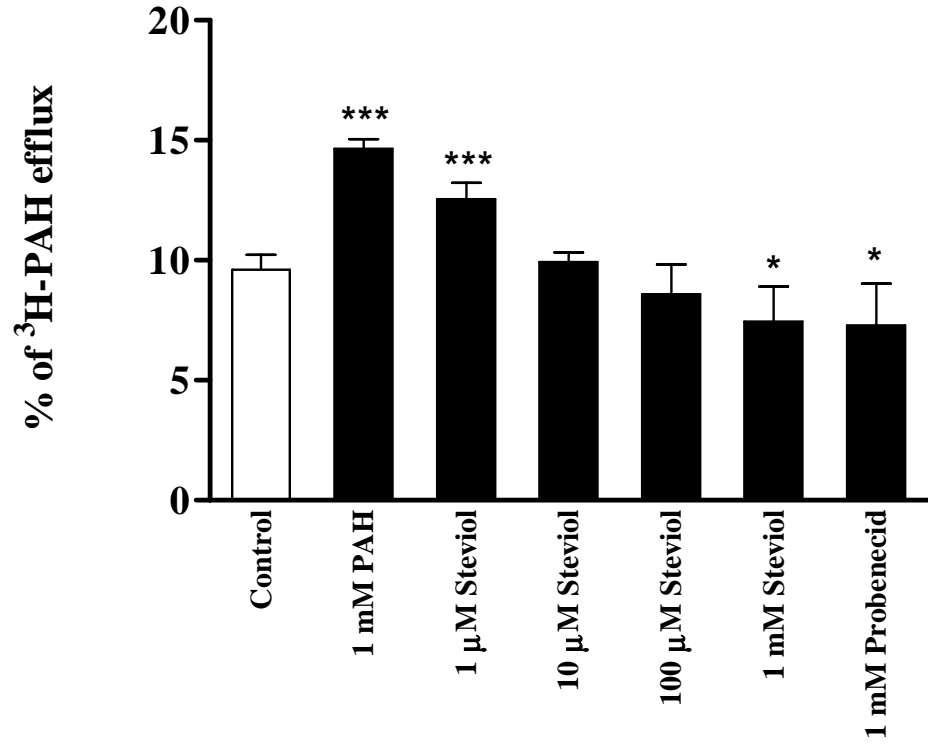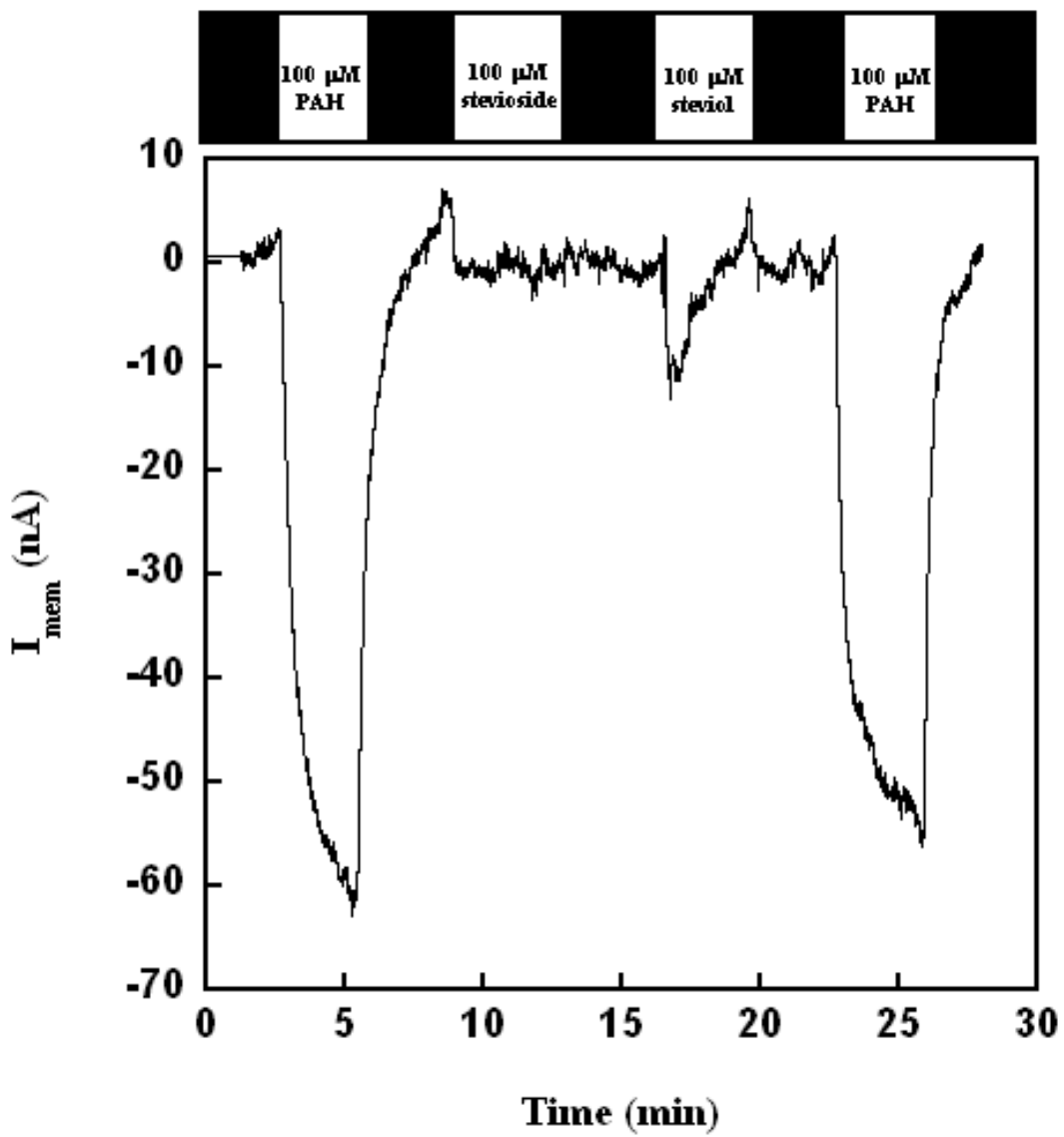
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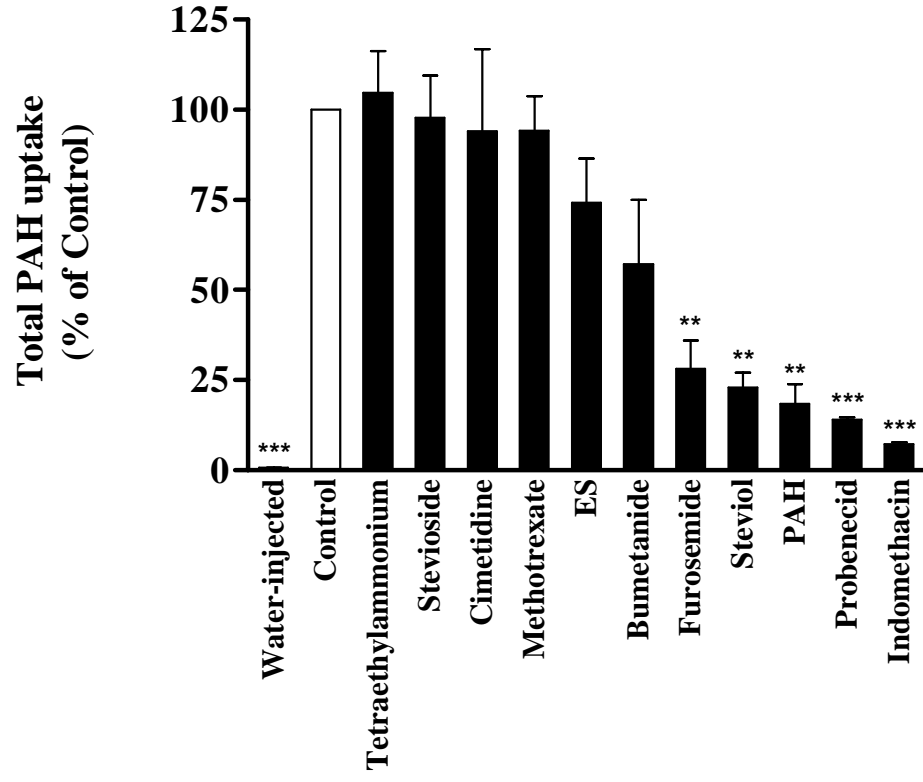
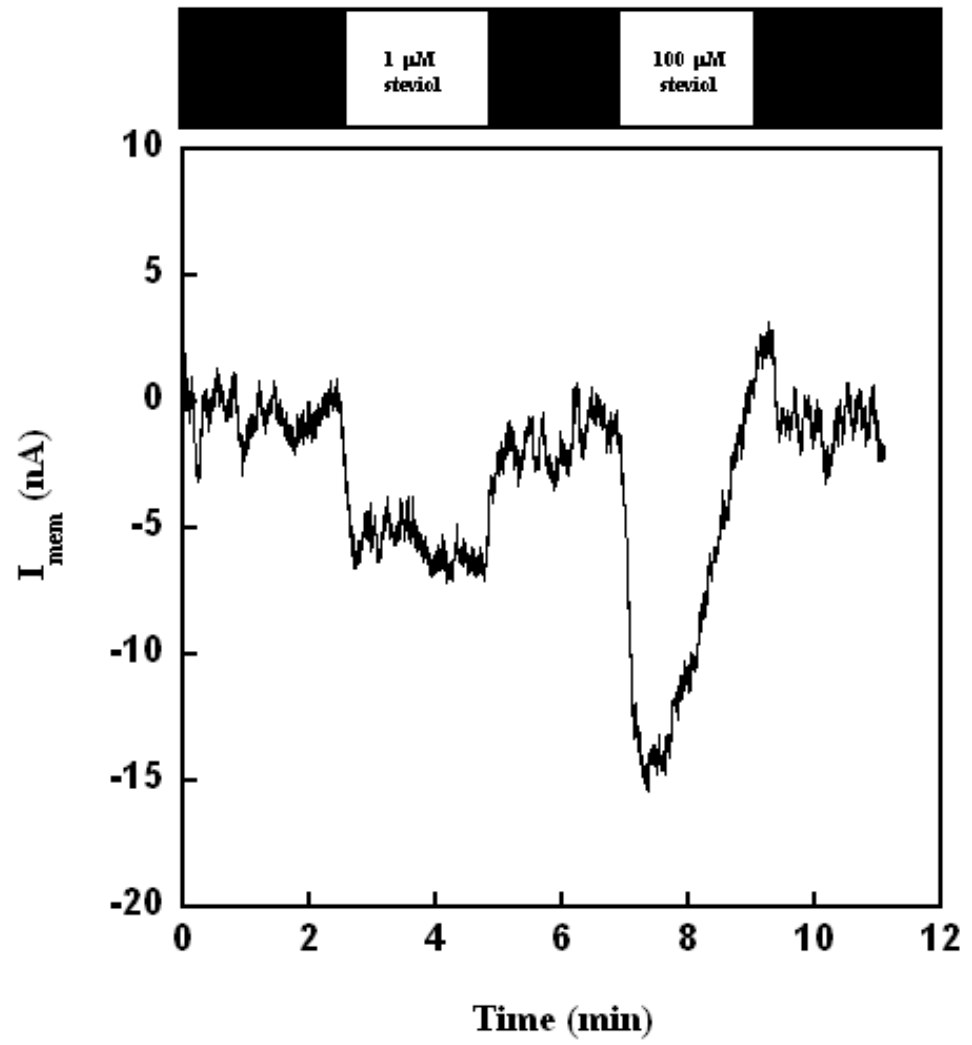Figure 2A
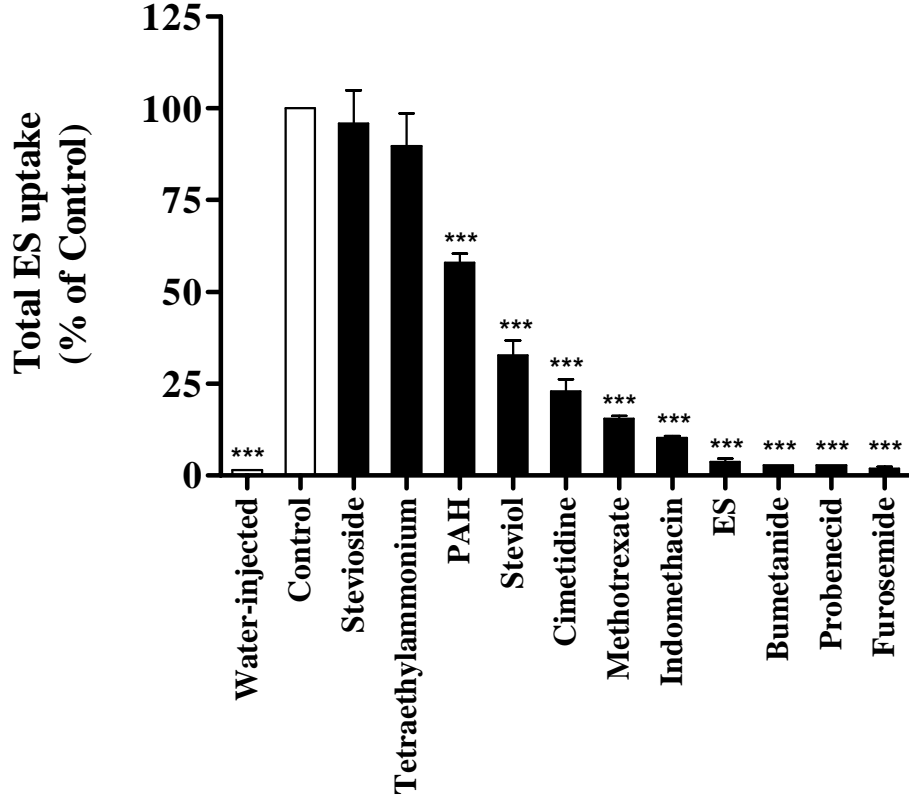
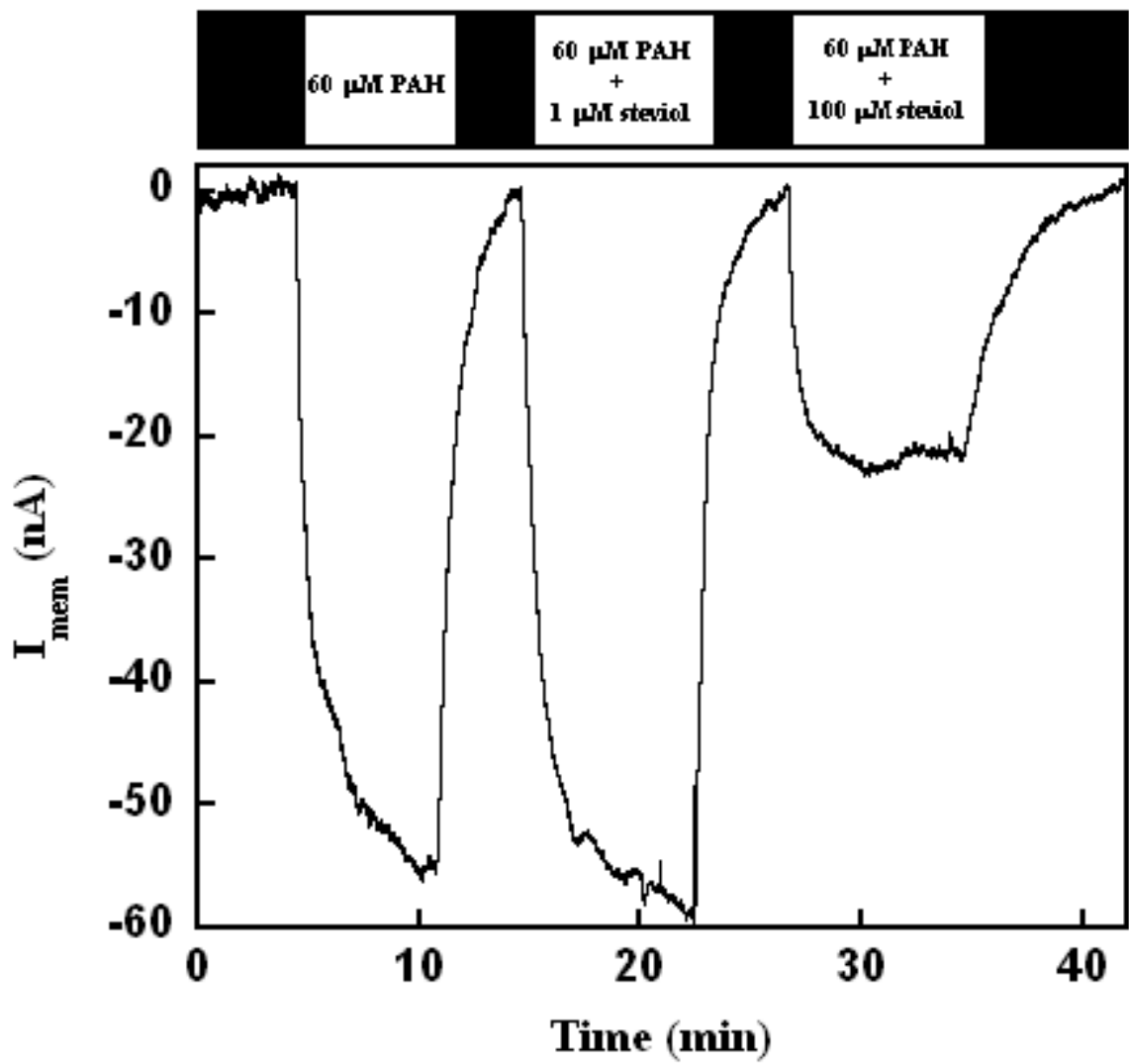Figure 2B
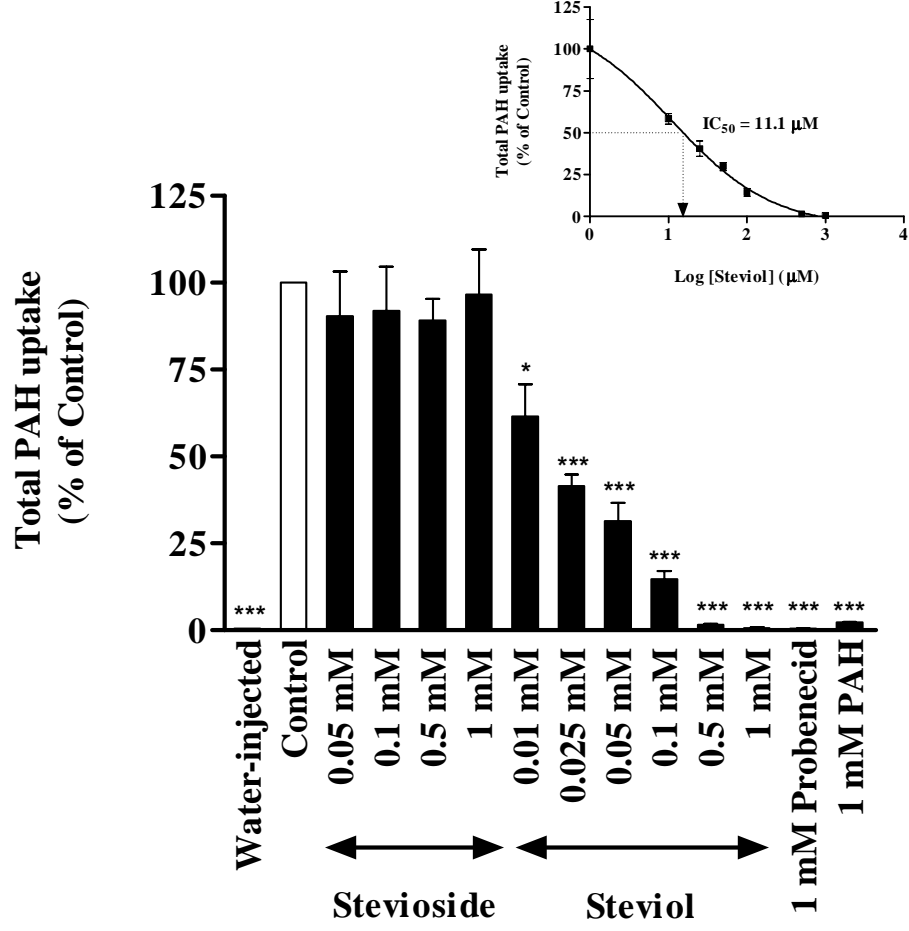
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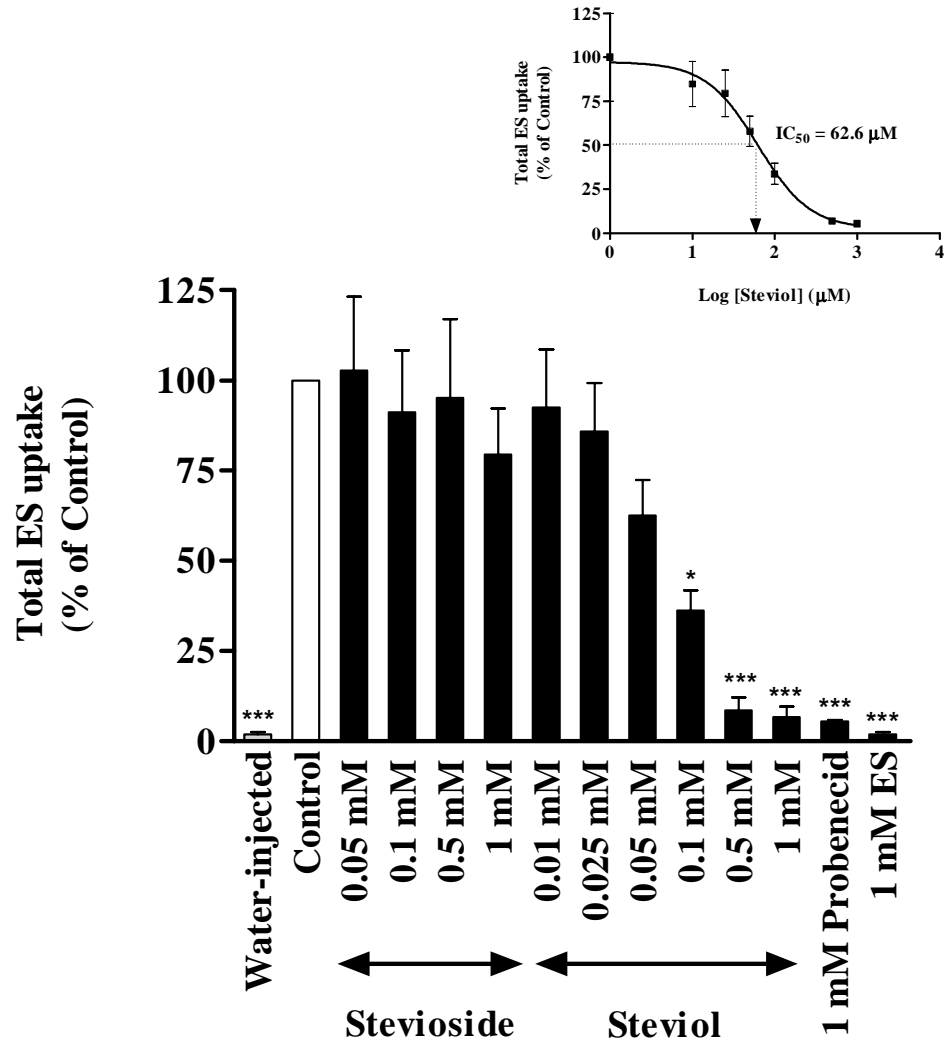
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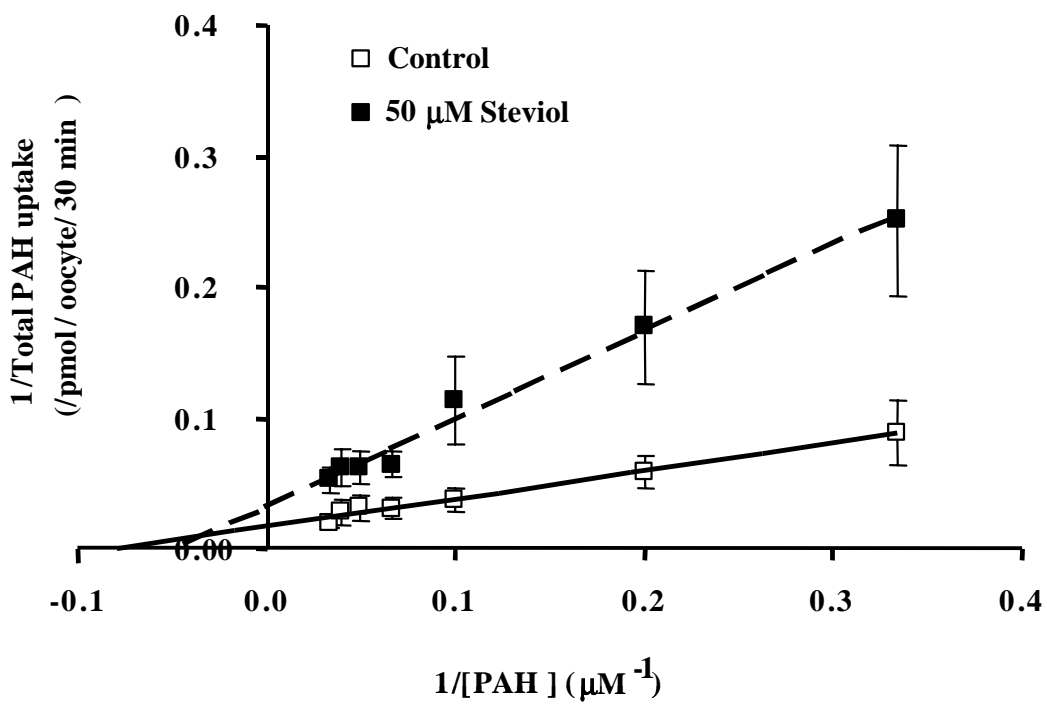
Figure 4A

Figure 4B

Figure 5A

Figure 5B

Figure 6A

Figure 6B

Figure 6C