DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



# Involvement of the $H^+$/organic cation antiporter in nicotine transport in rat liver.

Yuma Tega, Shin-ichi Akanuma, Yoshiyuki Kubo, Ken-ichi Hosoya

Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Japan

**Running title:** Carrier-mediated transport of nicotine in the liver.

**Corresponding author:** Professor Ken-ichi Hosoya, Ph.D.

Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.

Voice: +81-76-434-7505; FAX: +81-76-434-5172; E-mail: hosoyak@pha.u-toyama.ac.jp

Number of text pages: 17

Number of tables: 2

Number of figures: 1

Number of references: 19

Total words in the abstract: 264 words

Total words in the introduction: 507 words

Total words in the result and discussion: 847 words

**Abbreviations**

FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; LUI, liver uptake index; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



**Abstract**

Nicotine is an addictive alkaloid in cigarette smoke and is responsible for tobacco dependence. It is important to consider the blood-to-liver transport of nicotine in order to understand the nicotine elimination from the body since most of the nicotine is converted to inactive metabolites by cytochrome P450 localized in the endoplasmic reticulum of the hepatocytes. In this study, the blood-to-liver transport of nicotine was investigated by means of an *in vivo* portal vein injection technique in rats and the *in vitro* uptake by fleshly isolated rat hepatocytes was used to clarify its mechanism. The results obtained showed that the *in vivo* blood-to-liver transport of [$^3$H]nicotine was significantly inhibited by 50 mM nicotine and pyrilamine, suggesting involvement of a carrier-mediated transport process in the blood-to-liver transport of nicotine. The *in vitro* uptake study using freshly isolated rat hepatocytes showed a time- and concentration-dependent uptake of [$^3$H]nicotine with a $K_m$ value of 141 μM, and the uptake was increased under alkaline extracellular conditions. In addition, intracellular acidification caused an increase in [$^3$H]nicotine uptake, suggesting that the influx transport of nicotine is driven by an oppositely directed $H^+$ gradient in hepatocytes. Moreover, [$^3$H]nicotine uptake was strongly inhibited in the presence of cationic drugs, such as pyrilamine, whereas only weak inhibitory effects were shown by substrates of typical organic cation transporters (OCTs and OCTNs), such as tetraethylammonium, 1-methyl-4-phenylpyridinium, choline, and L-carnitine. In conclusion, a carrier-mediated system controlling the blood-to-liver transport of nicotine appears to be present on the sinusoidal membrane of hepatocytes. The pattern of inhibition and ion-dependence is suggestive of an $H^+$/organic cation antiporter-mediated nicotine transport system.

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



## Introduction

Smoking is an important risk factor in diseases such as cancer, cardiovascular disease, and chronic obstructive pulmonary disease. Nearly 6 million people die from smoking-related disease each year, and this number is expected to increase to more than 8 million by 2030 (Syed and Chaudhari, 2013), showing the great importance of smoking cessation to prevent increasingly severe health problems. Nicotine is a major alkaloid synthesized in *Nicotiana tabacum,* and is responsible for tobacco dependence. In the brain, nicotine stimulates dopamine release from the neurons via nicotinic acetylcholine receptors (nAChR) and causes feelings of pleasure and reward (Dani and De Biasi, 2001). In addition, nicotine causes withdrawal symptoms, such as irritability and anxiety, as consequence of a reduction in its concentration in the circulating blood (Hughes et al., 1984). Due to these neural events associated with nicotine, smokers inhale cigarette smoke repeatedly to maintain the nicotine concentration in their circulating blood, suggesting that obtaining information about the modulation of the nicotine concentration in the circulating blood will help improve the effectiveness of smoking cessation therapy.

Recently, our investigations revealed the involvement of a carrier-mediated transport process in the blood-to-brain transport of nicotine across the blood-brain barrier (BBB), based on the results obtained from *in vivo* and *in vitro* studies (Tega et al., 2013). In addition, carrier-mediated nicotine transport has also been reported in previous studies in Caco-2 cells and rat kidney (Fukada et al., 2002a; Fukada et al., 2002b), suggesting the important contribution of carrier-mediated transport to the tissue uptake of nicotine.

In the liver, it has been reported that 70-80% nicotine in the circulating blood is metabolized to cotinine by cytochrome P450 (CYP) (Benowitz and Jacob, 1994), showing that hepatic clearance plays an important role in nicotine elimination from the circulating blood. In humans, the genetic polymorphisms of CYP2A6, the hepatic enzyme involved in

nicotine metabolism (Nakajima et al., 2000), alter pharmacokinetics of nicotine, and individuals with a higher activity of CYP2A6 tend to develop tobacco dependence (Ray et al., 2009). In an *in vivo* study in mice lacking the CYP enzyme involved in nicotine metabolism, the pharmacokinetic parameters for nicotine, such as its half-life in blood and the area under the blood concentration-time curve, were altered to increase the sensitivity to rewarding effects of nicotine (Li et al., 2013). These lines of evidence strongly suggest that the hepatic clearance of nicotine affects the pharmacological response to nicotine. In terms of the interaction of nicotine with CYP, nicotine transport into intracellular space across the sinusoidal membrane of hepatocytes is essential for CYP-mediated metabolism of nicotine, since CYP is mainly localized in the endoplasmic reticulum of hepatocytes.

Therefore, clarification of the mechanism underlying nicotine uptake at the sinusoidal membrane of hepatocytes will provide helpful information about controlling the nicotine concentration in the circulating blood. In the present study, in order to examine the properties of the influx transport of nicotine by the liver, the nicotine transport was characterized using an *in vivo* portal vein injection technique called the liver uptake index (LUI) method and freshly isolated rat hepatocytes.

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



## Materials and Methods

### Animals and Reagents

Wistar rats (male, 150–200 g) were purchased from Japan SLC (Hamamatsu, Japan) and kept in a controlled environment. All experiments conformed to the provisions of the Animal Care Committee, University of Toyama. L-(-)-[N-methyl-$^{3}$H]Nicotine ([$^{3}$H]nicotine, 83.5 Ci/mmol) and [pyridinyl-5-$^{3}$H]pyrilamine ([$^{3}$H]pyrilamine, 20.0 Ci/mmol) were purchased from PerkinElmer (Boston, MA). *n*-[1-$^{14}$C]Butanol, ([$^{14}$C]*n*-butanol, 2 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were commercial products of analytical grade.

### Liver uptake index (LUI) method and cellular uptake study

The LUI method and a cellular uptake study involving freshly isolated hepatocytes were used to investigate the *in vivo* and *in vitro* transport of nicotine in the liver, respectively. The details are described in Supplemental Data.

### Statistical analysis

The kinetic parameters are presented as the means ± S.D. Other data are presented as the means ± S.E.M. To determine the significance of differences between two group means, an unpaired Student's t-test was used. To assess the statistical significance of differences among means of more than two groups, one-way ANOVA followed by Dunnett's test was used.

**Result and Discussion**

The *in vivo* hepatic uptake of [$^{3}$H]nicotine was 1.5-fold greater than that of [$^{14}$C]*n*-butanol, an internal reference. In the liver uptake index technique, the concentration of drugs in the space of Disse was assumed to be lower than that of the injectate by in part of mixing effect (Tsuji et al., 1989), and the inhibitors of high concentration (50 mM) were used. As the results, unlabeled nicotine and pyrilamine significantly reduced *in vivo* nicotine uptake in the liver while tetraethylammonium (TEA) and L-carnitine had little effect (Table 1). These results suggest that a pyrilamine-sensitive influx system is involved in blood-to-liver transport of nicotine.

In the *in vitro* analysis, [$^{3}$H]nicotine uptake exhibited time- and concentration-dependence with a $K_m$ of 141 μM, a $V_{max}$ of 1.78 nmol/(min·mg protein), and a $K_d$ of 5.69 μL/(min·mg protein), that were estimated from the uptake data obtained at 1 min (Fig. 1A and B), suggesting the involvement of a carrier-mediated transport process in nicotine uptake by rat hepatocytes. The $K_d$ was assumed to represent the non-saturable process including the non-specific nicotine adsorption exhibited by the y intercept (Fig. 1A), and/or passive diffusion of nicotine. In human, nicotine concentration in the circulating blood is reported to rise to 179-370 nM after smoking (Gourlay and Benowitz, 1997). Under the concentration range, the contribution of saturable component for total uptake clearance is estimated as 69%, suggesting the major role of carrier-mediated transport in hepatic nicotine uptake.

The study of ion-dependence suggests the extracellular $Na^+$- and membrane potential-independence of hepatic nicotine uptake because of the slight and insignificant changes in [$^{3}$H]nicotine uptake in buffer where $Na^+$ was replaced by $Li^+$ and $K^+$ (Fig. 1C). On the other hand, [$^{3}$H]nicotine uptake was reduced and increased at an extracellular pH of 6.4 and 8.4, respectively (Fig. 1D). Intracellular acidification increased [$^{3}$H]nicotine uptake, and

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



intracellular alkalization and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore, reduced the uptake (Fig. 1E and F), suggesting that hepatic nicotine transport is driven by an oppositely directed $H^+$ gradient.

The results of the inhibition study suggest the involvement of a cation-sensitive transport system in hepatic nicotine transport since the [$^3$H]nicotine uptake was strongly inhibited by hydrophobic cationic drugs, such as pyrilamine, verapamil, propranolol, quinidine, amantadine, and clonidine (Table 2). As the organic cation transporters, OCTs, OCTNs, MATEs, and PMAT are known (Engel and Wang, 2005; Gründemann et al., 1994; Hiasa et al., 2006; Kekuda et al., 1998;Tamai et al., 1997; Wu et al., 1998), however the substrates or inhibitors of organic cation transporters (OCTs, OCTNs, MATEs and PMAT) and organic anion transporters (OATs), such as TEA, L-carnitine, 1-methyl-4-phenylpyridinum ($MPP^+$), choline, and *p*-aminohippurate (PAH), slightly inhibited the uptake (Table 2), suggesting the involvement of a carrier-mediated transport process as distinct from the well-characterized transporters in hepatic nicotine transport.

Recently, the involvement of a putative pyrilamine transport system was suggested in the nicotine transport at the BBB (Okura et al., 2008; Tega et al., 2013), and the *in vitro* uptake study revealed that [$^3$H]pyrilamine uptake by isolated rat hepatocytes was strongly inhibited by cationic drugs, such as nicotine, but not substrates of typical organic cation transporters (Table 2). Although [$^3$H]pyrilamine uptake by isolated rat hepatocytes was exhibited time- and concentration-dependence with a $K_{m1}$ of 0.928 μM, a $V_{max1}$ of 0.533 nmol/(min·mg protein), a $K_{m2}$ of 351 μM, and a $V_{max2}$ of 34.2 nmol/(min·mg protein) (Supplemental Fig. 1A and B), these $K_m$ values are inconsistent with the $K_m$ (28 μM) of which pyrilamine uptake by TR-BBB13 cells, an *in vitro* rat BBB model (Okura et al., 2008). In addition, extracellular pH had no significant effect on [$^3$H]pyrilamine uptake by isolated rat hepatocytes (Supplemental Fig. 1C), during the pH-dependence shown in pyrilamine

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



uptake by TR-BBB13 cells (Okura et al., 2008). These results indicate that the transport mechanism of pyrilamine in the liver is different from putative pyrilamine transport system at the BBB, suggesting that the transport mechanism, distinct from putative pyrilamine transport system at the BBB, contributes to the nicotine transport in the liver.

In a kinetic study, nicotine uptake by isolated rat hepatocytes in the presence of pyrilamine exhibited a $K_m$ of 180 μM, a $V_{max}$ of 0.468 nmol/(min・mg protein), and a $K_d$ of 6.03 μL/(min・mg protein) (Supplemental Fig. 2), that suggested the non-competitive inhibition by pyrilamine in the liver since the $V_{max}$ was significantly reduced in the presence of pyrilamine in spite of the closely similar $K_m$ values. In our previous report, the competitive inhibition by pyrilamine was shown in nicotine uptake by TR-BBB13 cells (Tega et al., 2013), and these suggest that the nicotine uptake system in the liver is different from that of the BBB.

In conclusion, a carrier-mediated transport process involving the blood-to-liver transport of nicotine appears to be present on the sinusoidal membrane of the hepatocytes, and is suggested to be driven by the oppositely directed $H^+$ gradient. The inhibition study suggests that a novel transporter, highly sensitive to cationic drugs, contributes to nicotine transport by the liver. These findings provide helpful information to increase our understanding of the pharmacokinetics of nicotine.

**Authorship Contributions**

Participated in research design: Tega, Akanuma, Kubo and Hosoya

Conducted experiments: Tega

Contributed new reagents or analytic tools: N/A

Performed data analysis: Tega and Akanuma

Wrote or contributed to the writing of the manuscript: Tega, Akanuma, Kubo and Hosoya

**References**

Benowitz NL and Jacob P 3rd (1994) Metabolism of nicotine to cotinine studied by a dual stable isotope method. *Clin Pharmacol Ther* **56:** 483-493.

Dani JA and De Biasi M (2001) Cellular mechanisms of nicotine addiction. *Pharmacol Biochem Behav* **70:** 439-446.

Engel K and Wang J (2005) Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Mol Pharmacol* **68:** 1397-1407.

Fukada A, Saito H, and Inui K (2002a) Transport mechanisms of nicotine across the human intestinal epithelial cell line Caco-2. *J Pharmacol Exp Ther* **302:** 532–538.

Fukada A, Saito H, Urakami Y, Okuda M, and Inui K (2002b) Involvement of specific transport system of renal basolateral membranes in distribution of nicotine in rats. *Drug Metab Pharmacokinet* **17:** 554–560.

Gourlay SG and Benowitz NL (1997) Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. *Clin Pharmacol Ther* **62:** 453–463.

Gründemann D, Gorboulev V, Gambaryan S, Veyhl M, and Koepsell H (1994) Drug excretion mediated by a new prototype of polyspecific transporter. *Nature* **372:** 549-552.

Hiasa M, Matsumoto T, Komatsu T, and Moriyama Y (2006) Wide variety of locations for rodent MATE1, a transporter protein that mediates the final excretion step for toxic organic cations. *Am J Physiol Cell Physiol* **291:** C678-686.

Hughes JR, Hatsukami DK, Pickens RW, Krahn D, Malin S, and Luknic A (1984) Effect of nicotine on the tobacco withdrawal syndrome. *Psychopharmacology (Berl)* **83:** 82-87.

Kekuda R, Prasad PD, Wu X, Wang H, Fei YJ, Leibach FH, and Ganapathy V (1998) Cloning and functional characterization of a potential-sensitive, polyspecific organic cation

transporter (OCT3) most abundantly expressed in placenta. *J Biol Chem* **273:** 15971-15979.

Li L, Jia K, Zhou X, McCallum SE, Hough LB, and Ding X (2013) Impact of nicotine metabolism on nicotine's pharmacological effects and behavioral responses: insights from a Cyp2a(4/5)bgs-null mouse. *J Pharmacol Exp Ther* **347:** 746-754.

Nakajima M, Yamagishi S, Yamamoto H, Yamamoto T, Kuroiwa Y, and Yokoi T (2000) Deficient cotinine formation from nicotine is attributed to the whole deletion of the CYP2A6 gene in humans. *Clin Pharmacol Ther* **67:** 57-69.

Okura T, Hattori A, Takano Y, Sato T, Hammarlund-Udenaes M, Terasaki T, and Deguchi Y (2008) Involvement of the pyrilamine transporter, a putative organic cation transporter, in blood-brain barrier transport of oxycodone. *Drug Metab Dispos* **36:** 2005-2013.

Ray R, Tyndale RF, and Lerman C (2009) Nicotine dependence pharmacogenetics: role of genetic variation in nicotine-metabolizing enzymes. *J Neurogenet* **23:** 252-261.

Syed BA and Chaudhari K (2013) Smoking cessation drugs market. *Nat Rev Drug Discov* **12:** 97-98.

Tamai I, Yabuuchi H, Nezu J, Sai Y, Oku A, Shimane M, and Tsuji A (1997) Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. *FEBS Lett* **419:** 107-111.

Tega Y, Akanuma S, Kubo Y, Terasaki T, and Hosoya K (2013) Blood-to-brain influx transport of nicotine at the rat blood-brain barrier: Involvement of a pyrilamine-sensitive organic cation transport process. *Neurochem Int* **62:** 173–181.

Tsuji A, Terasaki T, Tamai I, and Takeda K (1990) In vivo evidence for carrier-mediated uptake of beta-lactam antibiotics through organic anion transport systems in rat kidney and liver. *J Pharmacol Exp Ther* **253:** 315-320.

Wu X, Prasad PD, Leibach FH, and Ganapathy V (1998) cDNA sequence, transport function,

and genomic organization of human OCTN2, a new member of the organic cation transporter family. *Biochem Biophys Res Commun* **246:** 589-595.

**Footnotes**

This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

**Figure Legends**

**Figure 1 Nicotine uptake by rat hepatocytes.** A, Time-dependent uptake of [$^3$H]nicotine (1 μCi/mL, 12 nM) was investigated for the indicated times. B, Concentration-dependent uptake of [$^3$H]nicotine was measured for 1 min, and the $K_m$, $V_{max,}$ and $K_d$ were estimated as 141 ± 63 μM, 1.78 ± 0.71 nmol/(min·mg protein), and 5.69 ± 0.67 μL/(min·mg protein), respectively. The solid and dashed lines represent the overall and saturable transport, respectively. C and D, Effect of $Na^+$, membrane potential and extracellular pH on [$^3$H]nicotine uptake was examined for 1 min. E, Intracellular pH-dependence on [$^3$H]nicotine uptake was examined for 15 sec. Acute treatment and pretreatment (Pre) with $NH_4Cl$ were used to make the intracellular pH alkaline and acidic, respectively. F, The cells were preincubated with or without 10 μM FCCP for 10 min, and subsequently [$^3$H]nicotine uptake was measured for 15 sec. These uptake studies were performed at 37°C. Each point or column represents the mean ± S.E.M. (n = 3-6). *$P$ < 0.05, **$P$ < 0.01, significantly different from control.

DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



**Table 1 Effect of inhibitors on *in vivo* [$^{3}$H]nicotine uptake by rat liver.** [$^{3}$H]Nicotine (1.8 μCi/rat) and [$^{14}$C]*n*-butanol (0.18 μCi/rat) dissolved in 200 μL Ringer-HEPES buffer (pH 7.4) were injected into the portal vein in the absence (control) or presence of inhibitors (50 mM). Each value represents the mean ± S.E.M. (n = 3-5). **P* < 0.05, ***P* < 0.01, significantly different from control.

| Compounds | LUI (%) | Percentage of control |
|---|---|---|
| Control | 150 ± 11 | 100 ± 7 |
| Nicotine | 84.3 ± 18.0** | 56.4 ± 12.0** |
| Pyrilamine | 111 ± 4* | 74.1 ± 2.7* |
| Tetraethylammonium (TEA) | 165 ± 9 | 110 ± 6 |
| L-Carnitine | 146 ± 4 | 97.4 ± 2.6 |

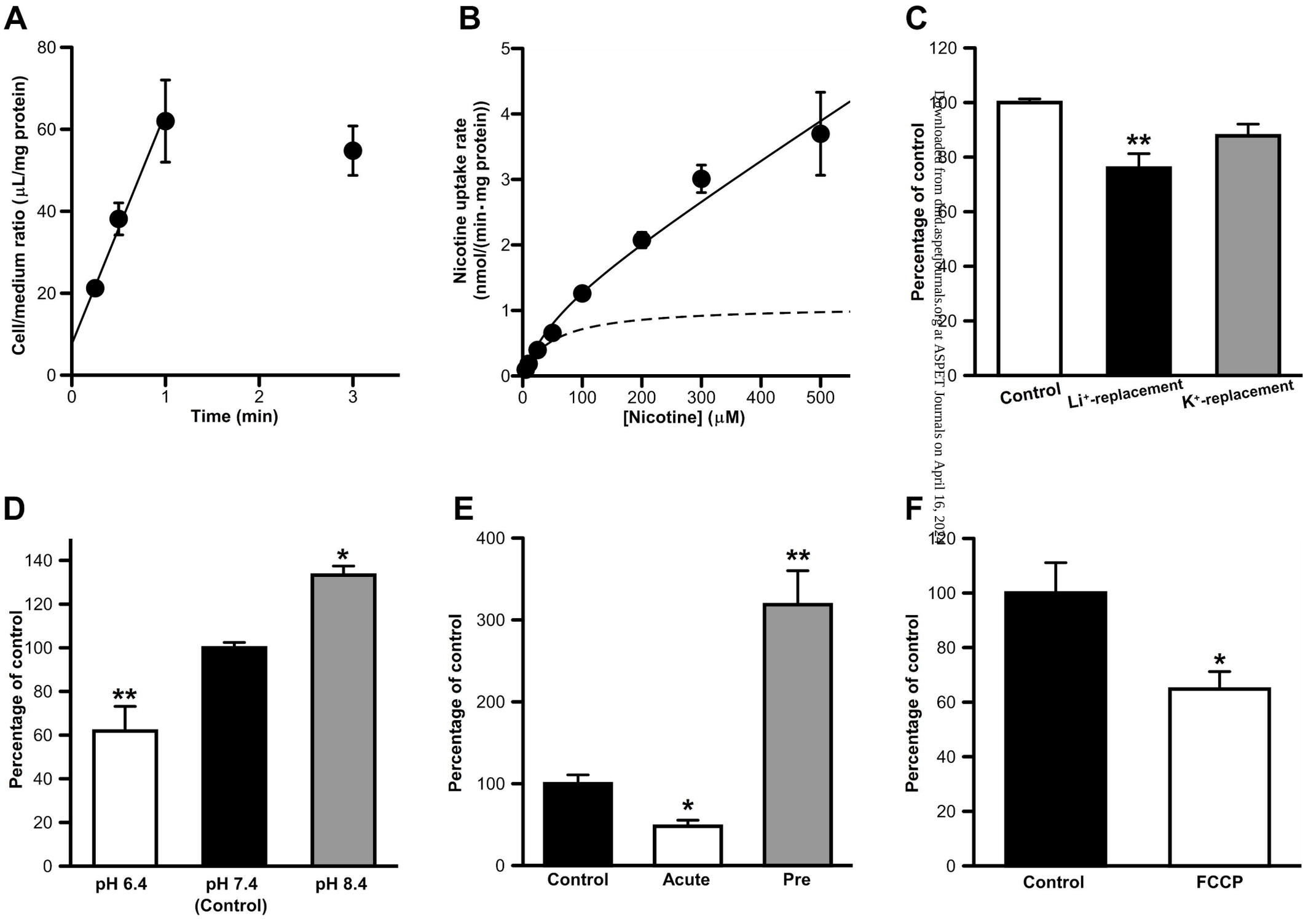
DMD Fast Forward. Published on October 28, 2014 as DOI: 10.1124/dmd.114.061002
This article has not been copyedited and formatted. The final version may differ from this version.



**Table 2 Inhibitory effect of several compounds on the uptake of [$^3$H]nicotine or [$^3$H]pyrilamine by rat hepatocytes.** The uptake of [$^3$H]nicotine (1 μCi/mL, 12 nM) and [$^3$H]pyrilamine (1 μCi/mL, 50 nM) was measured at 37°C for 1 min and 30 sec, respectively, in the absence (control) or presence of compounds (1 mM). Each value represents the mean ± S.E.M. (n = 3-6). ***P* < 0.01, significantly different from control.

| Compounds | [$^3$H]Nicotine | [$^3$H]Pyrilamine |
|---|---|---|
| | Percentage of control | Percentage of control |
| Control | 100 ± 2 | 100 ± 1 |
| Nicotine | 17.5 ± 0.5** | 9.34 ± 0.51** |
| Pyrilamine | 21.1 ± 0.8** | 4.60 ± 0.26** |
| Propranolol | 34.7 ± 0.9** | 2.46 ± 0.13 ** |
| Clonidine | 30.5 ± 1.0** | 12.4 ± 0.8** |
| Amantadine | 40.8 ± 1.1** | 15.9 ± 1.4** |
| Verapamil | 42.2 ± 1.0** | 5.29 ± 0.26** |
| Quinidine | 46.8 ± 1.3** | 3.74 ± 0.26** |
| 1-Methyl-4-phenylpyridinium ($MPP^+$) | 79.2 ± 2.8** | 73.2 ± 1.2** |
| Tetraethylammonium (TEA) | 79.5 ± 5.7** | 72.8 ± 2.1** |
| Choline | 95.8 ± 3.1 | 78.8 ± 2.0** |
| L-Carnitine | 121 ± 6** | 80.3 ± 0.4** |
| *p*-Aminohippurate (PAH) | 87.8 ± 3.0** | 79.4 ± 2.1** |

# Figure 1

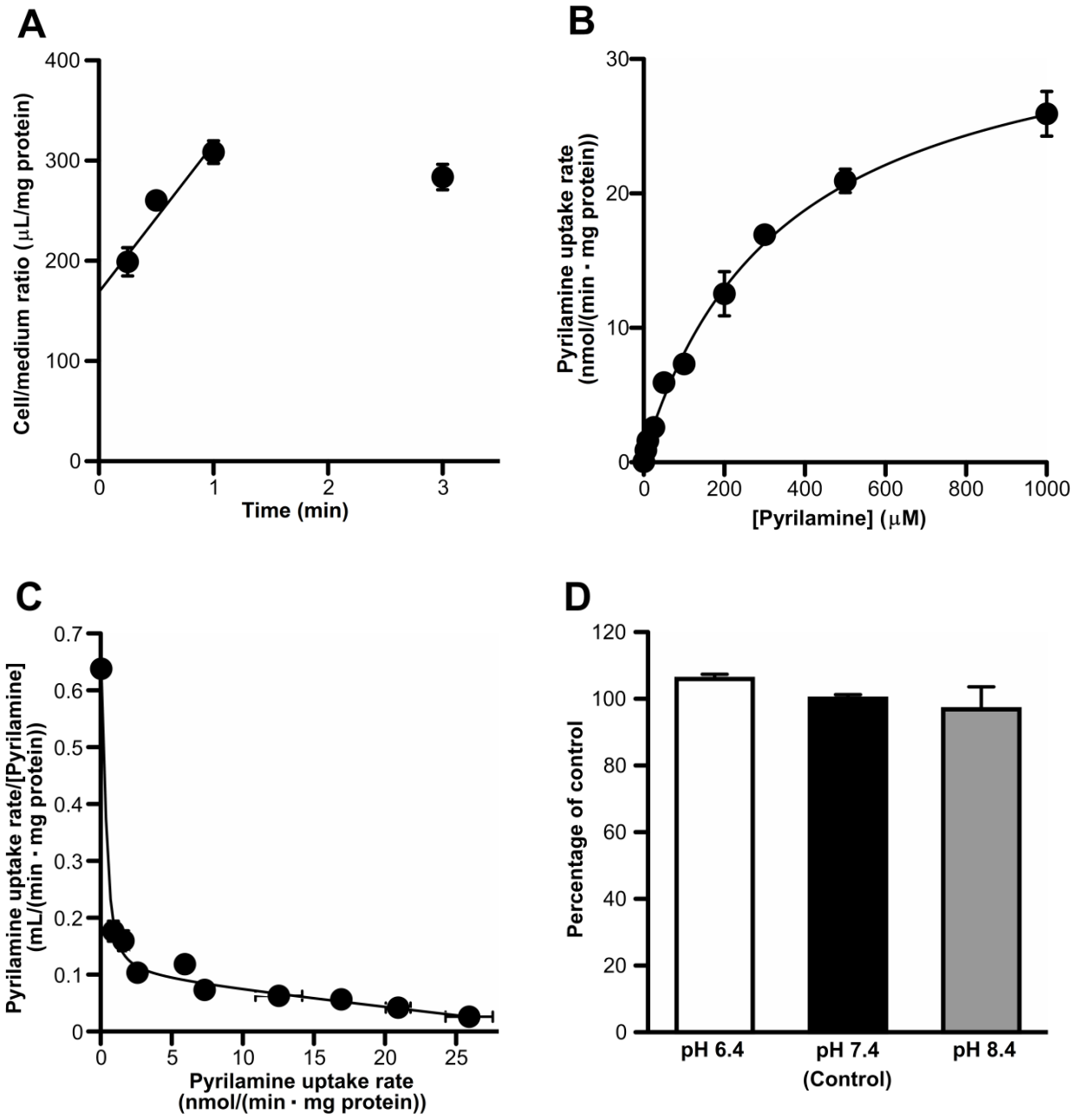


Supplemental Data

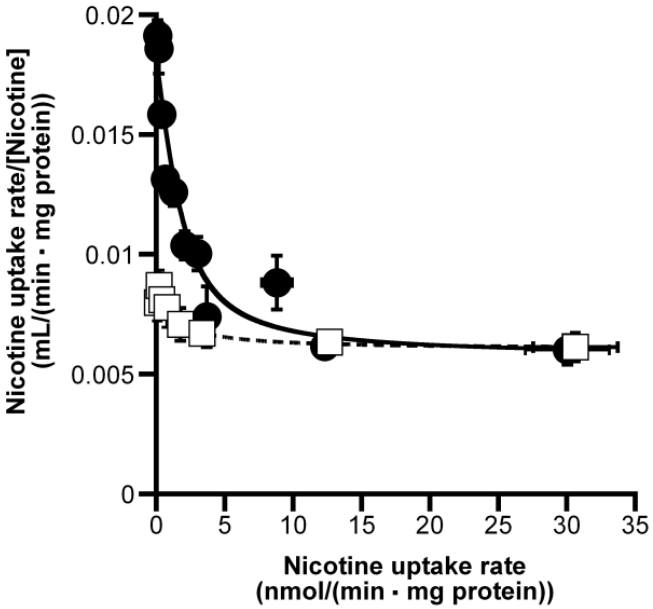
# Involvement of the $H^+$/organic cation antiporter in nicotine transport in rat liver.

Yuma Tega, Shin-ichi Akanuma, Yoshiyuki Kubo, Ken-ichi Hosoya

Drug Metabolism and Disposition




**Supplemental Fig. 1 [$^3$H]Pyrilamine uptake by rat hepatocytes.** A, Time-dependent uptake of [$^3$H]pyrilamine (1 μCi/mL, 50 nM) was measured at 37°C for the indicated times. B, Concentration-dependent uptake of [$^3$H]pyrilamine was measured at 37°C for 30 sec, and the $K_{m1}$, $V_{max1}$, $K_{m2}$, and $V_{max2}$ were estimated as 0.928 ± 0.326 μM, 0.533 ± 0.165 nmol/(min·mg protein), 351 ± 80 μM, and 34.2 ± 4.7 nmol/(min·mg protein), respectively. C, Eadie-Scatchard plot analysis of concentration-dependent uptake of [$^3$H]pyrilamine. D, Effect of extracellular pH on [$^3$H]pyrilamine uptake was examined at 37°C for 30 sec. Each point or column represents the mean ± S.E.M. (n = 3).




**Supplemental Fig. 2 Effect of pyrilamine on the concentration-dependent nicotine uptake by rat hepatocytes.** [$^3$H]Nicotine uptake (1 μCi/mL, 12 nM) was measured at 37°C for 1 min with increasing concentrations of nicotine in the absence (closed circles) or presence (open squares) of unlabeled pyrilamine (200 μM). The uptake in the presence of pyrilamine exhibited a $K_m$ of 180 ± 50 μM, a $V_{max}$ of 0.468 ± 0.087 nmol/(min · mg protein), and a $K_d$ of 6.03 ± 0.20 μL/(min · mg protein). Data were subjected to Eadie-Scatchard plot analysis. Each point represents the mean ± S.E.M. (n = 3-6).

## Supplemental Methods

### Liver uptake index (LUI) method

Nicotine uptake in the liver was determined by injecting [$^3$H]nicotine into the portal vein, as previously reported (Pardridge et al., 1979). Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and the hepatic artery was ligated. A solution (200 μL) containing [$^3$H]nicotine (1.8 μCi/200 μL, 108 nM) and [$^{14}$C]*n*-butanol (0.18 μCi/200 μL, 450 nM) as a highly diffusible internal reference dissolved in Ringer-HEPES buffer (141 mM NaCl, 4.0 mM KCl, 2.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4) in the absence or presence of inhibitors, was injected into the portal vein. The right major lobe was excised from the liver after 18 seconds and solubilized in Soluene-350 (PerkinElmer, Boston, MA). Then, 30% $H_2O_2$ was added to the solution to remove the color, followed by mixing with 10 mL Hionic-Fluor (PerkinElmer). The $^3$H and $^{14}$C radioactivity in the liver and the injection solutions were measured using a liquid scintillation spectrophotometer (LSC-5200; Aloka, Tokyo, Japan). [$^3$H]Nicotine uptake by the liver was described by eqn. 1:

$$\text{LUI (\%)} = ([^3\text{H}]/[^{14}\text{C}]\ (\text{dpm in the liver}))/([^3\text{H}]/[^{14}\text{C}]\ (\text{dpm in the injectate})) \times 100 \qquad (1)$$

### [$^3$H]Nicotine and [$^3$H]pyrilamine uptake by freshly isolated hepatocytes

As described previously (Berry et al., 1969; Petzinger et al., 1989), rat hepatocytes were isolated by collagenase perfusion and isodensity centrifugation in Percoll (Sigma, St. Louis, MO), and the [$^3$H]nicotine uptake by the freshly isolated hepatocytes was examined. The cell suspension ($4 \times 10^5$ cells) in Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4$, 5.6 mM D-glucose, pH 7.4) was centrifuged and the supernatant was aspirated. A solution containing [$^3$H]nicotine (0.1 μCi/100 μL, 12 nM) or [$^3$H]pyrilamine (0.1 μCi/100 μL, 50 nM) dissolved in Tyrode buffer was added to isolated hepatocytes and incubated at 37°C. When the influence of $Na^+$ was to be examined, $Na^+$ in Tyrode buffer was replaced with $Li^+$ involving the replacement of NaCl, $NaH_2PO_4$, and $NaHCO_3$ with LiCl, $KH_2PO_4$, and $KHCO_3$, respectively. When the influence of the membrane potential was to be examined, $Na^+$ in Tyrode buffer was replaced with $K^+$ involving the replacement of NaCl, $NaH_2PO_4$, and $NaHCO_3$ with KCl, $KH_2PO_4$, and $KHCO_3$, respectively. To terminate the uptake, cells were centrifuged in microfuge

tubes containing oil and 3N KOH layers. The cell-associated radioactivity and protein content were assayed by liquid scintillation counting (LSC-5200; Aloka) and a detergent-compatible protein assay (a DC protein assay kit; Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The cellular uptake was expressed as the cell/medium ratio (μL/mg protein) = ([$^3$H] dpm in the cell per cell protein (mg))/([$^3$H] dpm in the medium per medium volume (μL)).

The kinetic parameters for nicotine and pyrilamine uptake by hepatocytes were obtained from eqn. 2 and eqn. 3, respectively.

$$V = (V_{max} \times C)/(K_m + C) + K_d \times C \quad (2)$$

$$V = (V_{max1} \times C)/(K_{m1} + C) + (V_{max2} \times C)/(K_{m2} + C) \quad (3)$$

$V$ and $C$ are the uptake rate and the medium concentration of each substrate, respectively. In eqn. 2, $V_{max}$, $K_m$, and $K_d$ are the maximum uptake rate, the Michaelis-Menten constant, and the non-saturable transport clearance of nicotine, respectively. In eqn. 3, $V_{max1}$, $V_{max2}$, $K_{m1}$, and $K_{m2}$ are the maximum uptake rate for the high-affinity process, the maximum uptake rate for the low-affinity process, the Michaelis-Menten constant for the high-affinity process, and the Michaelis-Menten constant for the low-affinity process of pyrilamine, respectively. To obtain kinetic parameters, the equation was fitted using the iterative nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981).

When examining the extracellular pH-dependence, the medium pH value was changed to 6.4, 7.4, and 8.4. To determine the influence of the $H^+$ gradient, 10 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore, was treated for 10 min, and subsequently [$^3$H]nicotine uptake was measured at pH 7.4. In the study of intracellular pH-dependence, $NH_4Cl$ at a concentration of 20 mM was used to alter the intracellular pH of hepatocytes (Gleeson et al., 1989). To acidify the intracellular pH, the cells were preincubated with Tyrode buffer with replaced $K^+$ (pH 7.4) in the presence of $NH_4Cl$ for 10 min and subsequently in Tyrode buffer with replaced $K^+$ ($NH_4Cl$-free) for 5 min, because intracellular $NH_3$ rapidly diffuses out of cells, resulting in the accumulation of $H^+$ released from $NH_4^+$ in a process to produce $NH_3$ (Ohta et al., 2006).

**References**

Berry MN and Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* **43:** 506-520.

Gleeson D, Smith ND and Boyer JL (1989) Bicarbonate-dependent and -independent intracellular pH regulatory mechanisms in rat hepatocytes. Evidence for $Na^+$-$HCO_3$- cotransport. *J Clin Invest* **84:** 312–321.

Ohta KY, Inoue K, Hayashi Y and Yuasa H (2006) Molecular identification and functional characterization of rat multidrug and toxin extrusion type transporter 1 as an organic cation/H+ antiporter in the kidney. *Drug Metab Dispos* **34:** 1868–1874.

Pardridge WM and Mietus LJ (1979) Transport of protein-bound steroid hormones into liver *in vivo*. *Am J Physiol Endocrinol Metab* **237:** E367–372.

Petzinger E, Müller N, Föllmann W, Deutscher J and Kinne RK (1989) Uptake of bumetanide into isolated rat hepatocytes and primary liver cell cultures. *Am J Physiol Gastrointest Liver Physiol* **256:** G78–86.

Yamaoka K, Tanigawara Y, Nakagawa T and Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4:** 879–885.