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

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Abbreviations

FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; LUI, liver uptake index; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter
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 freshly isolated rat hepatocytes was used to clarify its mechanism. The results obtained showed that the in vivo blood-to-liver transport of [3H]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 [3H]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 [3H]nicotine uptake, suggesting that the influx transport of nicotine is driven by an oppositely directed $H^+$ gradient in hepatocytes. Moreover, [3H]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.
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.
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-3H]Nicotine ([3H]nicotine, 83.5 Ci/mmol) and [pyridinyl-5-3H]pyrilamine ([3H]pyrilamine, 20.0 Ci/mmol) were purchased from PerkinElmer (Boston, MA). n-[1-14C]Butanol, ([14C]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 \textit{in vivo} hepatic uptake of $[^3\text{H}]$nicotine was 1.5-fold greater than that of
$[^{14}\text{C}]\mu$-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 \textit{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 \textit{in vitro} analysis, $[^3\text{H}]$nicotine uptake exhibited time- and
concentration-dependence with a $K_m$ of 141 \textmu M, a $V_{\text{max}}$ of 1.78 nmol/(min$\cdot$mg protein), and a
$K_d$ of 5.69 \textmu L/(min$\cdot$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 $[^3\text{H}]$nicotine uptake in buffer where Na$^+$ was replaced by Li$^+$ and K$^+$ (Fig. 1C). On
the other hand, $[^3\text{H}]$nicotine uptake was reduced and increased at an extracellular pH of 6.4
and 8.4, respectively (Fig. 1D). Intracellular acidification increased $[^3\text{H}]$nicotine uptake, and
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 $[^3H]$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-phenylpyridinium (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 $[^3H]$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 $[^3H]$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 $[^3H]$pyrilamine uptake by isolated rat hepatocytes (Supplemental Fig. 1C), during the pH-dependence shown in pyrilamine
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 $V_{\text{max}}$ of 0.468 nmol/(min $\cdot$ mg protein), and a $K_d$ of 6.03 µL/(min $\cdot$ mg protein) (Supplemental Fig. 2), that suggested the non-competitive inhibition by pyrilamine in the liver since the $V_{\text{max}}$ 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


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

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Figure Legends

Figure 1 Nicotine uptake by rat hepatocytes. A, Time-dependent uptake of [³H]nicotine (1 µCi/mL, 12 nM) was investigated for the indicated times. B, Concentration-dependent uptake of [³H]nicotine was measured for 1 min, and the $K_m$, $V_{max}$, and $K_d$ were estimated as $141 \pm 63$ µM, $1.78 \pm 0.71$ nmol/(min·mg protein), and $5.69 \pm 0.67$ 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 [³H]nicotine uptake was examined for 1 min. E, Intracellular pH-dependence on [³H]nicotine uptake was examined for 15 sec. Acute treatment and pretreatment (Pre) with NH₄Cl 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 [³H]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.
Table 1 Effect of inhibitors on in vivo [3H]nicotine uptake by rat liver. [3H]Nicotine (1.8 μCi/rat) and [14C]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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LUI (%)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150 ± 11</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Nicotine</td>
<td>84.3 ± 18.0**</td>
<td>56.4 ± 12.0**</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>111 ± 4*</td>
<td>74.1 ± 2.7*</td>
</tr>
<tr>
<td>Tetraethylammonium (TEA)</td>
<td>165 ± 9</td>
<td>110 ± 6</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>146 ± 4</td>
<td>97.4 ± 2.6</td>
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</table>
Table 2 Inhibitory effect of several compounds on the uptake of \([^3\text{H}]\)nicotine or \([^3\text{H}]\)pyrilamine by rat hepatocytes. The uptake of \([^3\text{H}]\)nicotine (1 µCi/mL, 12 nM) and \([^3\text{H}]\)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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>([^3\text{H}])Nicotine</th>
<th>([^3\text{H}])Pyrilamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 2</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Nicotine</td>
<td>17.5 ± 0.5**</td>
<td>9.34 ± 0.51**</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>21.1 ± 0.8**</td>
<td>4.60 ± 0.26**</td>
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<tr>
<td>Propranolol</td>
<td>34.7 ± 0.9**</td>
<td>2.46 ± 0.13 **</td>
</tr>
<tr>
<td>Clonidine</td>
<td>30.5 ± 1.0**</td>
<td>12.4 ± 0.8**</td>
</tr>
<tr>
<td>Amantadine</td>
<td>40.8 ± 1.1**</td>
<td>15.9 ± 1.4**</td>
</tr>
<tr>
<td>Verapamil</td>
<td>42.2 ± 1.0**</td>
<td>5.29 ± 0.26**</td>
</tr>
<tr>
<td>Quinidine</td>
<td>46.8 ± 1.3**</td>
<td>3.74 ± 0.26**</td>
</tr>
<tr>
<td>1-Methyl-4-phenylpyridinium (MPP+)</td>
<td>79.2 ± 2.8**</td>
<td>73.2 ± 1.2**</td>
</tr>
<tr>
<td>Tetraethylammonium (TEA)</td>
<td>79.5 ± 5.7**</td>
<td>72.8 ± 2.1**</td>
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<tr>
<td>Choline</td>
<td>95.8 ± 3.1</td>
<td>78.8 ± 2.0**</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>121 ± 6**</td>
<td>80.3 ± 0.4**</td>
</tr>
<tr>
<td>(p)-Aminohippurate (PAH)</td>
<td>87.8 ± 3.0**</td>
<td>79.4 ± 2.1**</td>
</tr>
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</table>
Supplemental Data

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

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Drug Metabolism and Disposition
Supplemental Fig. 1[^3H]Pyrilamine uptake by rat hepatocytes. A, Time-dependent uptake of[^3H]pyrilamine (1 µCi/mL, 50 nM) was measured at 37°C for the indicated times. B, Concentration-dependent uptake of[^3H]pyrilamine was measured at 37°C for 30 sec, and the $K_m$, $V_{max}$, $K_m$, and $V_{max}$ 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[^3H]pyrilamine. D, Effect of extracellular pH on[^3H]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 $180 \pm 50$ µM, a $V_{max}$ of $0.468 \pm 0.087$ nmol/(min · mg protein), and a $K_d$ of $6.03 \pm 0.20$ 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 $[^3\text{H}]$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 $[^3\text{H}]$nicotine (1.8 µCi/200 µL, 108 nM) and $[^14\text{C}]n$-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$_2$O$_2$ was added to the solution to remove the color, followed by mixing with 10 mL Hionic-Fluor (PerkinElmer). The $^3\text{H}$ and $^{14}\text{C}$ radioactivity in the liver and the injection solutions were measured using a liquid scintillation spectrophotometer (LSC-5200; Aloka, Tokyo, Japan). $[^3\text{H}]$Nicotine uptake by the liver was described by eqn. 1:

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

$[^3\text{H}]$Nicotine and $[^3\text{H}]$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 $[^3\text{H}]$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$_2$PO$_4$, 5.6 mM D-glucose, pH 7.4) was centrifuged and the supernatant was aspirated. A solution containing $[^3\text{H}]$nicotine (0.1 µCi/100 µL, 12 nM) or $[^3\text{H}]$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$_2$PO$_4$, and NaHCO$_3$ with LiCl, KH$_2$PO$_4$, 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$_2$PO$_4$, and NaHCO$_3$ with KCl, KH$_2$PO$_4$, 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) = ([³H] dpm in the cell per cell protein (mg))/([³H] 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.

\[ V = \frac{V_{\text{max}} \times C}{K_m + C} + K_d \times C \]  \hspace{1cm} (2)
\[ V = \frac{V_{\text{max1}} \times C}{K_{m1} + C} + \frac{V_{\text{max2}} \times C}{K_{m2} + C} \]  \hspace{1cm} (3)

\( V \) and \( C \) are the uptake rate and the medium concentration of each substrate, respectively. In eqn. 2, \( V_{\text{max}}, K_m, \) and \( K_d \) are the maximum uptake rate, the Michaelis-Menten constant, and the non-saturable transport clearance of nicotine, respectively. In eqn. 3, \( V_{\text{max1}}, V_{\text{max2}}, K_{m1}, \) 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 [³H]nicotine uptake was measured at pH 7.4. In the study of intracellular pH-dependence, NH₄Cl 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 NH₄Cl for 10 min and subsequently in Tyrode buffer with replaced K⁺ (NH₄Cl-free) for 5 min, because intracellular NH₃ rapidly diffuses out of cells, resulting in the accumulation of H⁺ released from NH₄⁺ in a process to produce NH₃ (Ohta et al., 2006).
References


