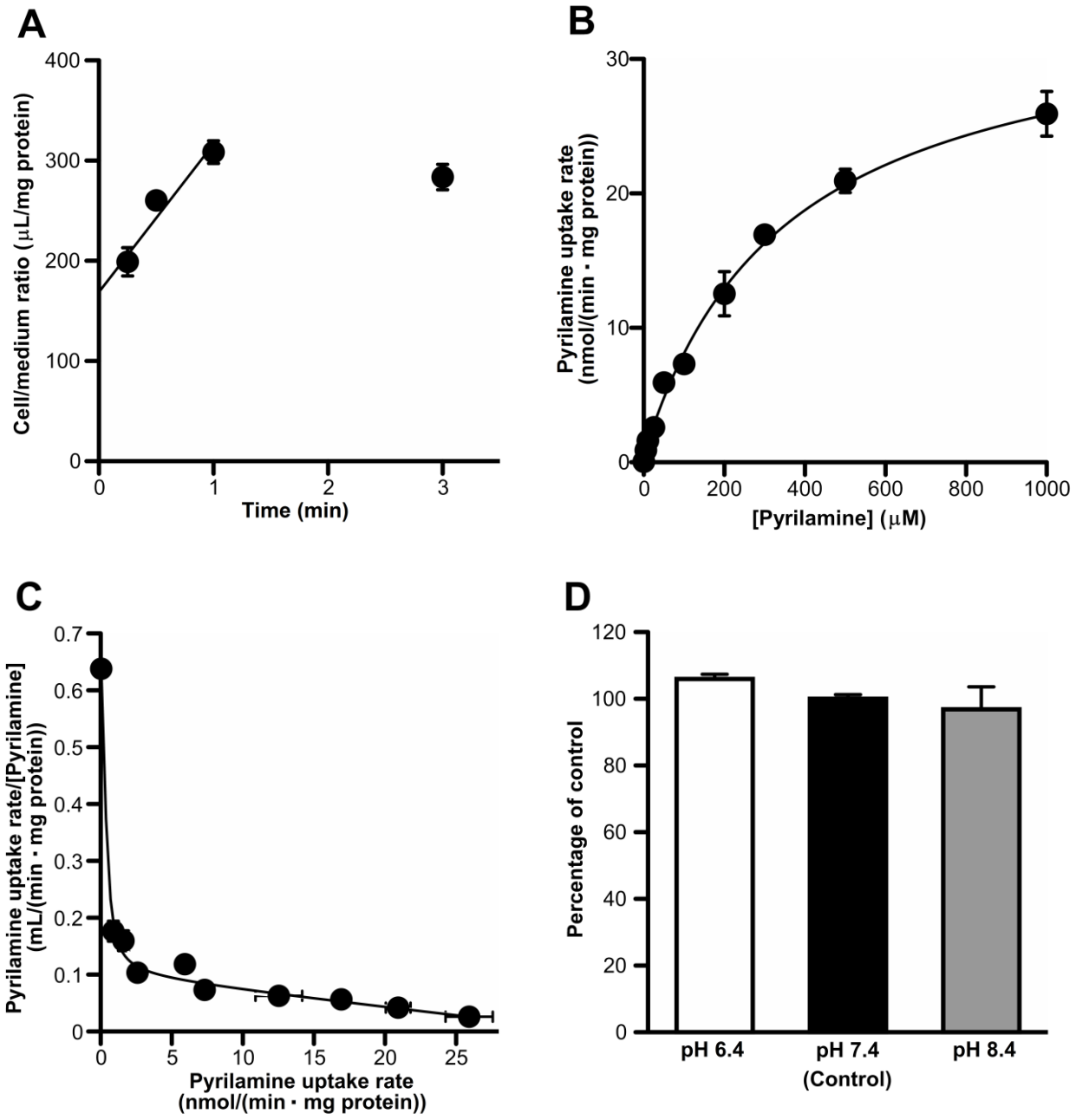
Supplemental Data

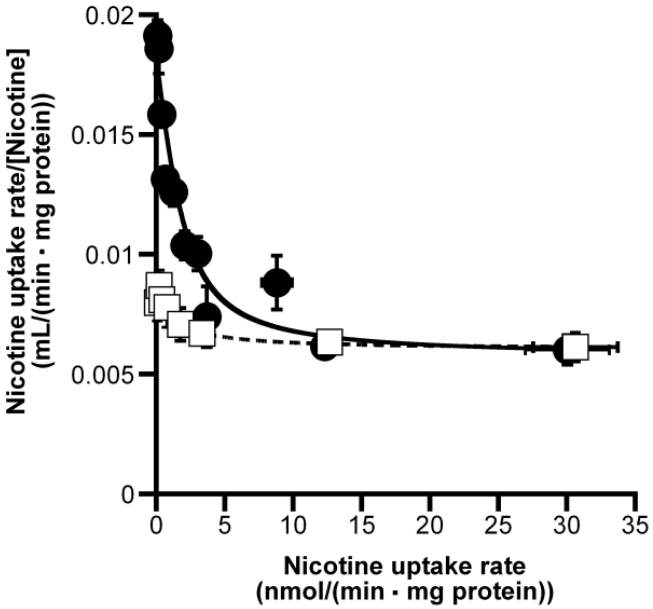
Involvement of the H^+/organic cation antiporter in nicotine transport in rat liver.

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 (4×10^5 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.