Transporter-Mediated Renal Handling of Nafamostat Mesilate

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ABSTRACT: Nafamostat mesilate (NM) is a serine-protease inhibitor that is rapidly eliminated from the circulation and accumulated in the kidney. This study was conducted to characterize the mechanism of NM transport in the kidney because a serious side effect of NM-induced hyperkalemia may be related to accumulation of NM in the kidney. Measurements of uptake of NM *in vivo* by the kidney uptake index (KUI) method and of transport in an *in vitro*-cultured LLC-PK1 cell system suggested the involvement of an organic cation transporter (OCT). To clarify the involvement of OCTs located in the basolateral membrane of proximal tubules, we evaluated NM transport by OCTs expressed in *Xenopus laevis* oocytes. The IC_{50} values of NM on [^{14}C]TEA ([^{14}C]tetraethylammonium) uptake by rOCT1, rOCT2, and hOCT2 were 50, 0.5, and 20 μM, respectively, and NM was concluded to be a substrate of OCTs. To investigate the transport of NM across the brush-border membrane, we examined the uptake of NM into brush-border membrane vesicles (BBMVs) isolated from rat renal cortex. NM was taken up into the BBMVs, and the uptake was decreased by unlabeled NM and temperature, implying that a transporter(s) is also involved in NM transport across the apical membrane. NM was not a substrate of hOCTN1, hOCTN2, or P-gp, implying the involvement of some unknown transporter(s). Thus, renal accumulation of NM can be explained by the involvement of the basolateral OCTs, though the influence of the apical membrane transporter remains to be clarified. J Pharm Sci 93:262–272, 2004

Keywords: renal transport; organic cation transporters; nafamostat mesilate

INTRODUCTION

Nafamostat mesilate (NM), an organic cationic compound, is an ester conjugate of *p*-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN). NM is a serine-protease inhibitor[1] that is used for the treatment of acute pancreatitis[2] and disseminated intravascular coagulation (DIC), and as an anticoagulant during hemodialysis.[3,4]

NM has also been reported to cause hyperkalemia and/or hyponatremia in rare cases after continuous intravenous infusion for the treatment of pancreatitis or DIC.[5] These side effects of NM were proposed to be associated in part with an inhibition of amiloride-sensitive Na^+ conductance (ENaC) in the apical membrane of the cortical collecting duct (CCD); Na^+-Cl^- co-transport by ENaC was blocked, resulting in a reduced influx of K^+ from blood to epithelial cells via Na^+/K^+ ATPase.[6,7] Recently, the reduction of prostasin urinary excretion was also reported to decrease the activity of ENaC, as evidenced by the decrease in the urinary excretion of prostasin accompanied by a significant increase in the urinary Na^+/K^+ ratio following adrenalectomy.[8] These results suggest that prostasin is involved, at least in part, in hyperkalemia and/or hyponatremia. Prostasin is a glycosyl-phosphatidylinositol (GPI)-anchored

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protein that is expressed in the cortex and medulla of the kidney,[9] but is not found in the membrane fraction or cytosol fraction of the kidney.[10] Thus, an intracellular proteolytic mechanism that could result in the selective and efficient secretion of soluble forms of folate receptors has been suggested.[11] NM has been shown to block the urinary secretion of prostasin and sodium, whereas PGBA and AN have little effect.[10] NM was also found in the kidney at a high concentration for a long period and was not observed in urine, but it disappeared rapidly from plasma after intravenous administration in rats.[12] Therefore, it is suggested that accumulation of NM in the kidney blocks the urinary secretion of prostasin and consequently inhibits the prostasin-induced sodium uptake through ENaC, resulting in the side effects of hyperkalemia and/or hyponatremia. However, the mechanism of renal handling of NM is poorly understood. Therefore, the aim of this study was to clarify the mechanism of NM transport in the kidney.

Renal organic cation transport in proximal tubules and distal tubules has been investigated in various species and is considered to reflect the coordinated actions of the transporters located in the basolateral and brush-border membranes of the tubular cells.[13–15] Secretion of cationic compounds consists of two steps; they are, uptake by tubular epithelial cells across the basolateral membrane and transport across the brush-border membrane. In the basolateral membrane of proximal tubules, organic cation transporters OCT1 and OCT2 are involved in the transport of organic cationic drugs in human and rat kidney.[16,17] In brush-border membrane, OCTN1[18,19] and OCTN2[20,21] were reported to be involved in organic cation transport, in addition to P-glycoprotein (P-gp), which transports various cationic xenobiotics as an efflux pump.[22,23]

In this study, we examined the kidney uptake of NM by the kidney uptake index (KUI) method *in vivo*, and in proximal tubule LLC-PK1 cells *in vitro*. To characterize the NM uptake system(s), we used *Xenopus laevis* oocytes expressing rOCT1, rOCT2, and hOCT2. In addition, to investigate the transport of NM in the apical membrane of the renal cortex, we examined the uptake of NM into brush-border membrane vesicles (BBMVs) isolated from renal cortex of rats. To clarify the involvement of cation transporters other than those of the OCT family, such as hOCTN1, hOCTN2, and P-gp, the inhibitory effects on the uptake of typical substrates of hOCTN1 and hOCTN2 were measured using HEK293 cells that stably express these transporters. In addition, NM transport experiments were performed using cultured monolayers of LLC-GA5-COL150 cells, a cultured cell model that over expresses P-gp.

EXPERIMENTAL

Materials

[Ethyl-1-^{14}C]tetraethylammonium bromide (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). *p*-[Glycyl-2-^{3}H]-aminohippuric acid (3.55 Ci/mmol), [^{3}H]daunomycin (18.5 Ci/mmol), and [^{3}H]water (1 mCi/g) were obtained from NEN Life Science Products Inc. (Boston, MA). L-[Methyl-^{3}H]carnitine hydrochloride (81 Ci/mmol) was purchased from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, UK). [^{14}C]Nafamostat mesilate ([^{14}C]NM, 52 mCi/mmol), nafamostat mesilate (NM), *p*-guanidinobenzoic acid (PGBA), and 6-amidino-2-naphthol (AN) were supplied by Torii Pharmaceutical Company, Ltd. (Tokyo, Japan).

Animals

Six- to seven-week-old male Wistar rats, weighing 200–220 g, were purchased from Japan SLC (Hamamatsu, Japan). Mature female *Xenopus laevis* were purchased from Hamamatsu Kyozai (Hamamatsu, Japan) and maintained in a controlled environment.[24] Animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University.

Cell Culture

LLC-PK1 and LLC-GA5-COL150 cells[25,26] obtained from Riken Cell Bank (Tsukuba, Japan) were cultured and grown on Transwells™, and the transport experiments were performed by the Transwell method, as described previously.[27] The confluent cells were washed with Hanks' balanced salt solution [HBSS, 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), pH7.4], and 0.5 mL and 1.5 mL of HBSS

were added on the apical and basolateral sides, respectively, of a cell insert. To measure transcellular transport, a test compound was included in either the apical or basolateral side. At the designated times, samples (0.5 mL of basolateral-side solution or 0.2 mL of apical-side solution) were withdrawn from the acceptor compartment and replaced with an equal volume of HBSS. The radioactivity in each solution was measured.

For the uptake experiments in suspension, LLC-PK1 cells were isolated in HBSS. After pre-incubation at 37°C for 15 min, the uptake was initiated by adding an aliquot of 200 μL of the drug solution containing [^{14}C]TEA ([^{14}C]tetraethylammonium) to 200 μL of LLC-PK1 cell suspension. The uptake was terminated by centrifugation at 13,000 rpm for 30 s, and the supernatant fluids were discarded. The pellets were washed with ice-cold HBSS, then the cells were centrifuged again at 13,000 rpm for 30 s. The radioactivity of the cell pellets was measured.

HEK293 cells stably expressing hOCTN1 and hOCTN2 were established and cultured, and uptake experiments were performed for 3 min as described previously.[17,19] The cells were harvested and suspended in transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (pH 7.4). After pre-incubation of the cell suspension at 37°C for 10 min, transport experiments were initiated by mixing the cell suspension with a solution of a radiolabeled test compound in the transport medium. At appropriate times, 200-μL aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicon oil and liquid paraffin with a density of 1.03. Each cell pellet was solubilized in 3*N* KOH and neutralized with HCl, and then the associated radioactivity was measured.

Kidney Uptake Index (KUI)

An abdominal aortal injection was performed as described in previous reports.[28,29] Rats were anesthetized with diethyl ether. Five seconds after the rapid artery injection, the entire kidney was removed. About 100–150 mg of renal cortex tissue was solubilized in 2.0 mL of Soluene-350 at 55°C for 3 h. Then, 600 μL of hydrogen peroxide, 200 μL of 5*N* hydrochloric acid, and 8 mL of Clearsol I (Nacalai Tesque) were added, before the double-isotope radioactivity was measured with a liquid scintillation counter. The KUI was determined with eq. 1[28,29]:

$$\mathrm{KUI}\,(\%) = \frac{\left([^{14}\mathrm{C}]/[^{3}\mathrm{H}]\mathrm{H_2O}\,\mathrm{dpm}\right)\text{ in kidney}}{\left([^{14}\mathrm{C}]/[^{3}\mathrm{H}]\mathrm{H_2O}\,\mathrm{dpm}\right)\text{ in injection solution}} \times 100 \quad (1)$$

cRNA Transcription

Capped cRNAs of rOCT1 and rOCT2 were synthesized with T7 polymerase (mCAP RNA capping kit; Stratagene, La Jolla, CA) from plasmids donated by Dr. V. Ganapathy (Medical College of Georgia, Atlanta, GA. The capped cRNAs of hOCT2 were synthesized with SP6 polymerase (RiboMAX^{TM} Large Scale RNA Production Systems-SP6; Promega, Madison, WI) from plasmids purchased from ResGen^{TM} Invitrogen Corporation (Invitrogen Japan K.K., Tokyo, Japan).

Uptake Measurements in *Xenopus laevis* Oocytes

Oocytes were harvested from *Xenopus laevis* as described previously.[30] Three days after injection of 50 nL of cRNA (25 ng) or water, the oocytes were placed in a 24-well microtiter plate (10–14 oocytes into each well) and pre-incubated at room temperature for 5–10 minutes in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2{\cdot}6H_2O$, 5 mM HEPES, pH 7.4). The uptake of [^{14}C]TEA or [^{14}C]NM was measured in ND96 solution. Oocytes were rapidly rinsed five times with ice-cold ND96 solution, to terminate the uptake, and then incubated for 2 h in individual scintillation vials containing 0.1 mL of 5% sodium dodecyl sulfate (SDS). The radioactivity was measured with a liquid scintillation counter.

Transport Study in Brush Border Membrane Vesicles (BBMVs)

BBMVs were isolated from the renal cortex of rats using a calcium precipitation method.[31] The purity of BBMVs was assessed by measuring the activity of a marker enzyme (alkaline phosphatase) for brush-border membrane with respect to the initial homogenate. The protein concentration was 11.3 ± 0.41 mg of protein per milliliter. Transport activities of BBMVs were also confirmed by assessing the uptake of D-[^{3}H]glucose as

described previously.[31] Then, uptake of [^{14}C]NM by BBMVs was examined by a rapid filtration technique, as described previously.[31] The influx was initiated by adding an aliquot of 90 μL of the solution (100 mM mannitol, 100 mM NaCl, 20 mM HEPES/Tris, pH 7.5) containing [^{14}C]NM to 10 μL of membrane suspension. The uptake was terminated by adding 1 mL of ice-cold stop solution (300 mM mannitol and 20 mM HEPES/Tris, pH 7.5), and the mixture was immediately filtered through a Millipore filter (Millipore Ltd., Bedford, MA; HAWP, 0.45 μm) under vacuum. The filter was rapidly washed twice with 4 mL of ice-cold stop solution, and the radioactivity of the filter was measured.

Analytical Methods

Cellular protein content was determined according to the method of Bradford with a BioRad (Hercules, CA) protein assay kit with bovine serum albumin as the standard.[32] Nonradioactive nafamostat in the transport experiments was measured by high-performance liquid chromatography (HPLC). The HPLC system consisted of a constant-flow pump (PU 980, Japan Spectroscopic Company, Tokyo, Japan), an ultraviolet (UV) detector (UV 975, Japan Spectroscopic Company, Tokyo, Japan), an integrator (Chromatopac CR7A, Shimadzu Company, Japan), and an automatic sample injector (AS-1555-10, Japan Spectroscopic Company, Tokyo, Japan). An YMC-pack ODS-M80 column (150 × 4.6 mm, YMC Company, Ltd., Kyoto, Japan) was used, and kept at 40°C. The mobile phase consisted of 0.1 M acetic acid containing 0.01 M 1-heptanesulfonic acid sodium salt and acetonitrile (75:25), and the flow rate was 1.5 mL/min. The injection volume was 20 μL, and the eluate was monitored at 254 nm.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM), and statistical analysis was performed by the Student's *t*-test. A different between means was considered to be significant when the p value was <0.05.

RESULTS

Uptake of [^{14}C]NM into the Kidney *In Vivo*

Uptake of NM into the kidney for 5 s was measured by the KUI method. The values of percent uptake in kidney of [^{14}C]NM and [^{14}C]*p*-aminohippurate ([^{14}C]PAH) relative to that of [^{3}H]H_2O were 387.1 ± 26.6 and 313.8 ± 19.8, respectively (Table 1). Coadministration of TEA, a typical substrate of organic cation transporters, significantly decreased the uptake of [^{14}C]NM, but did not affect the uptake of [^{14}C]PAH, a typical substrate of organic anion transporters (Table 1). Therefore, these results imply a possibility that an organic cation transport system(s) is involved in NM uptake across the basolateral membrane of proximal tubular cells.

Table 1. Effects of Tetraethylammonium (TEA) on Renal Uptake of [^{14}C]NM and [^{14}C]PAH after Aortic Injection[a]

	Kidney Uptake Index (%)	
Treatment	[^{14}C]NM	[^{14}C]PAH
Control	387.1 ± 26.6	313.8 ± 19.8
+TEA	307.9 ± 15.3[b]	324.0 ± 16.9

[a]Each value represents the mean ± SEM ($n = 4–8$ rats per mean) of percent kidney (renal cortex) influx of ^{14}C-labeled NM or PAH relative to $^{3}H_2O$, in groups of control rats and those coadministered with unlabeled TEA (20 mM in injection solution).

[b]$P < 0.05$ significantly from control.

Transport of NM by LLC-PK1 Cells, a Proximal Tubular Epithelial Cell Line

LLC-PK1 cells were used to examine the uptake of NM because this cell line has been widely used as a model to investigate organic cation transport systems.[33] The time-dependent uptake of NM was measured using LLC-PK1 cells in suspension. A suspension method was used because the ability to simultaneously measure the uptake of NM from either basolateral membrane or apical membrane is efficient. The steady-state uptake of NM into LLC-PK1 cells in suspension was 87.3 ± 6.9 μL/mg protein for 120 min (Fig. 1A), whereas the cellular volume of LLC-PK1 cells was 12.5 ± 0.9 μL/mg protein as calculated from the [^{3}H]H_2O uptake minus that of [^{14}C]inulin, an extracellular marker. Thus, NM was apparently taken up concentratively by LLC-PK1 cells because the amount of steady-state uptake of NM was larger than the cellular volume.

The concentration-dependent uptake of [^{14}C]TEA (4.6 μM) in the presence or absence of unlabeled NM at 0.5 μM is shown in Figure 1B.

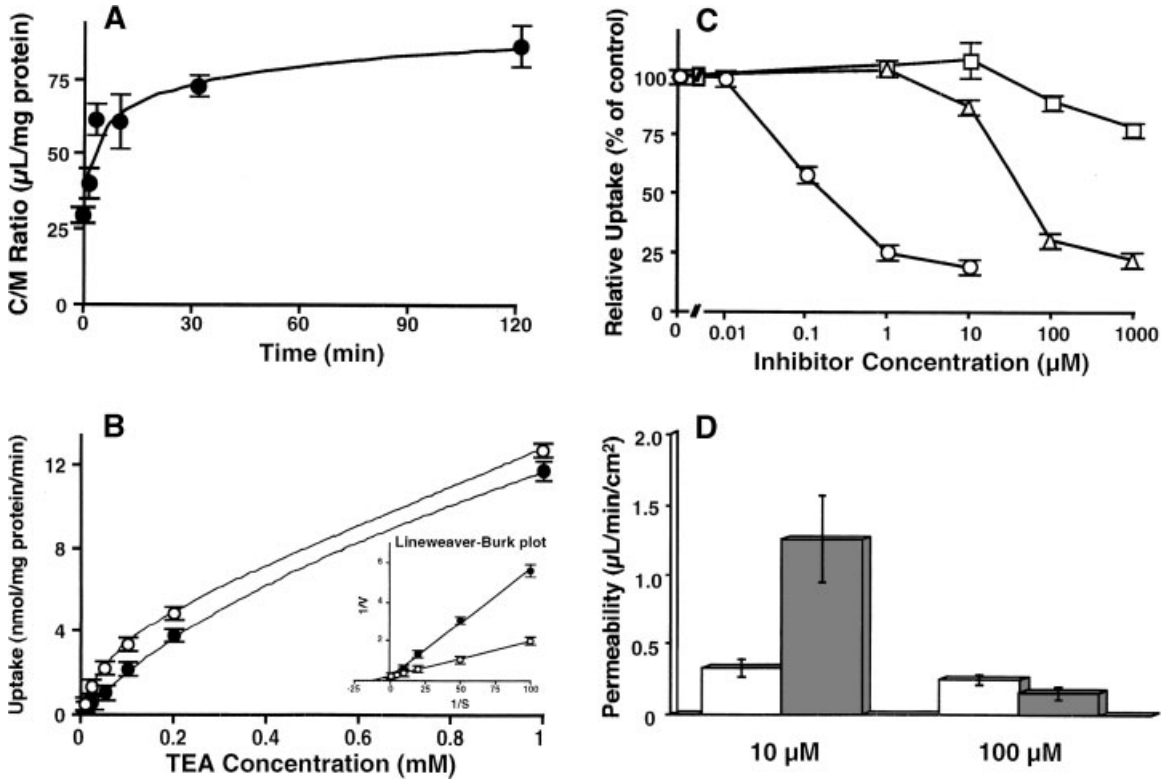

Figure 1. NM transport in proximal tubular epithelial cell line LLC-PK1. LLC-PK1 cells were suspended in HBSS at pH 7.0 (A, B, and C) or cultured as monolayers (D). (A) Uptake of NM (10 μM) was examined at 37°C, pH 7.0, for 120 min. (B) Uptake of [^{14}C]TEA (4.6 μM) was measured at 37°C, pH 7.0, for 1 min in the absence (open) or presence (closed) of NM (0.5 μM). Each point represents the mean ± SEM of three or four experiments. (C) Uptake of [^{14}C]TEA (4.6 μM) was measured at 37°C, pH 7.0, for 1 min in the presence of various concentrations of NM (○), AN (△), and PGBA (□). (D) Permeability of NM at the initial concentration of 10 or 100 μM was measured at 37°C, for 60 min, in HBSS (pH_{apical}/$pH_{basolateral}$ = 7.0/7.0) across cultured monolayers of LLC-PK1 cells. Open bars and closed bars represent transport in the apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions, respectively.

Michaelis–Menten analysis revealed that the addition of 0.5 μM NM increased the Michaelis constant (K_m) for TEA from 74.5 ± 4.3 to 661 ± 285 μM, but did not significantly change the values of maximum velocity (V_{max}; 4.32 ± 0.25 versus 11.4 ± 6.7 nmol/mg protein; Fig. 1B). Therefore, it implied that NM possibly inhibited the uptake of [^{14}C]TEA in a competitive manner. To characterize the transporters involved in NM uptake, the inhibitory effects of NM and its metabolites on the uptake of [^{14}C]TEA (4.6 μM) by LLC-PK1 cells in suspension were examined (Fig. 1C). Uptake of [^{14}C]TEA was inhibited by NM as well as its two metabolites to different extents, and the IC_{50} values (concentration giving 50% inhibition) of NM and its metabolites, AN, and PGBA, were estimated to be about 0.1 μM, 50 μM, and >1 mM, respectively. Based on the hypothesis that NM inhibited the uptake of [^{14}C]TEA in a competitive manner, the K_i value was estimated to be 0.06 μM.

The transcellular transport of NM across cultured monolayers of LLC-PK1 cells in the basolateral-to-apical (B-to-A) and apical-to-basolateral (A-to-B) directions was measured at different initial concentrations and was linear over the time course examined (60 min). A directional permeation of NM was observed at lower initial concentration (10 μM), the B-to-A transport being higher than the A-to-B transport, but this was not seen at the higher concentration (100 μM; Fig. 1D). Thus, the B-to-A transport was decreased significantly at higher initial concentration, suggesting the involvement of saturable transport system(s) in NM transport in the efflux direction.

Involvement of OCTs Located at the Basolateral Membrane of Proximal Tubular Cells

To clarify the involvement of OCTs, *Xenopus laevis* oocytes expressing rOCT1, rOCT2, and hOCT2 were used. We examined the inhibitory effect of NM on [^{14}C]TEA uptake, and the observed values of IC_{50} values were about 50, 0.5, and 20 μM for rOCT1, rOCT2, and hOCT2, respectively (Fig. 2A–C). The time course of [^{14}C]NM uptake was examined at a lower initial concentration than these values of IC_{50}. [^{14}C]NM uptake mediated by these OCTs was linear over the time course examined, and significantly higher than that of *Xenopus laevis* oocytes injected with water (Fig. 2D–F). These results clearly demonstrate that NM is a substrate of rOCT1, rOCT2, and hOCT2.

Characteristics of NM Transport Across the Brush Border Membrane of Renal Cortex

To investigate the transport of NM across apical membrane of proximal tubular cells, we examined the uptake of [^{14}C]NM using BBMVs isolated from rat renal cortex. The amount of [^{14}C]NM uptake into BBMVs was higher at 37°C than that at 25 or 4°C, and [^{14}C]NM uptake was significantly inhibited by unlabeled NM (Fig. 3A). These results imply that a transport system(s) is also involved in NM transport across the apical membrane of proximal tubules. The effect of pH in the outer medium on the uptake of [^{14}C]NM was examined, and the uptake of [^{14}C]NM in pH 6.0 was lower than that in pH 7.0 (data not shown).

To check the amount of surface binding on BBMVs, we examined the effect of osmolarity in

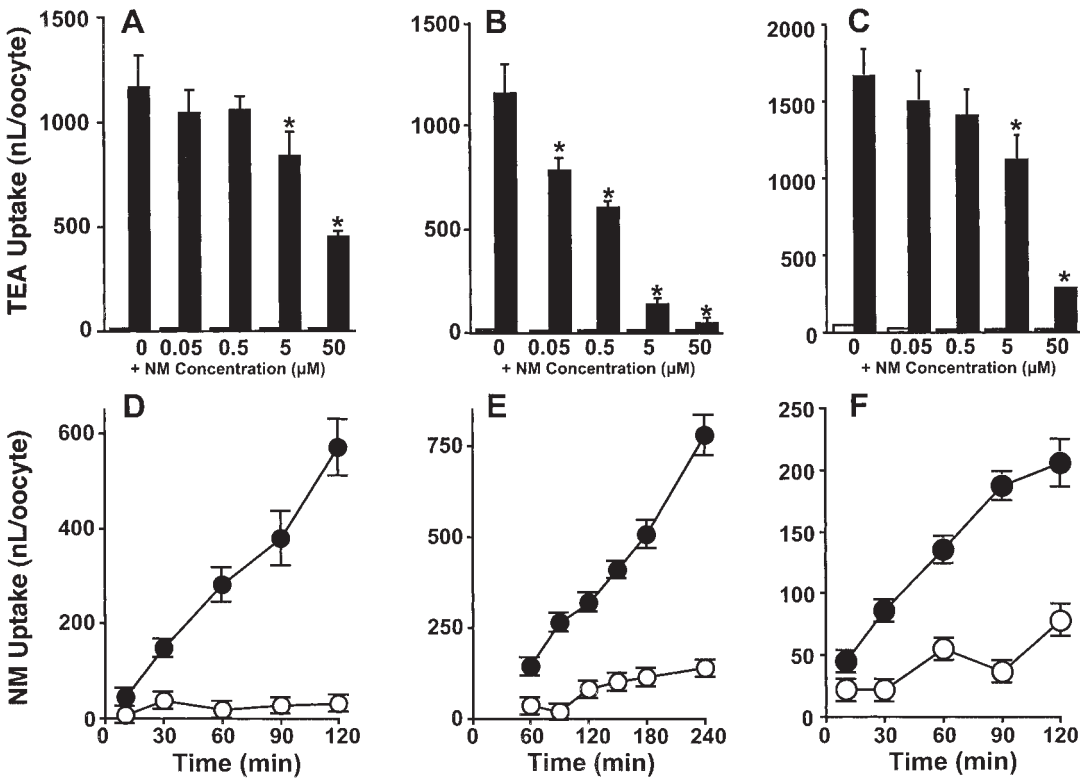

Figure 2. Transport of NM by rOCT1, rOCT2, and hOCT2 expressed in *Xenopus laevis* oocytes. Three days after injection of *Xenopus laevis* oocytes with 50 nL of rOCT1 (A and D), rOCT2 (B and E), and hOCT2 (C and F), cRNA (25 ng), or water, uptake of [^{14}C] NM and [^{14}C]TEA were measured at 25°C, pH 7.6. Uptake of [^{14}C]TEA (72 μM) was measured in the presence of various concentrations of NM (from 0.05 to 50 μM, A, B and C) for 60 min, using *Xenopus laevis* oocytes injected with OCT cRNA (closed bar) or water (open bar). The time course of [^{14}C]NM uptake (D, 30 μM; E, 1.5 μM; F, 3.0 μM) was determined using *Xenopus laevis* oocytes injected with OCTs cRNA (●) or water (○). Each column or point represents the mean ± SEM of eight or ten experiments.

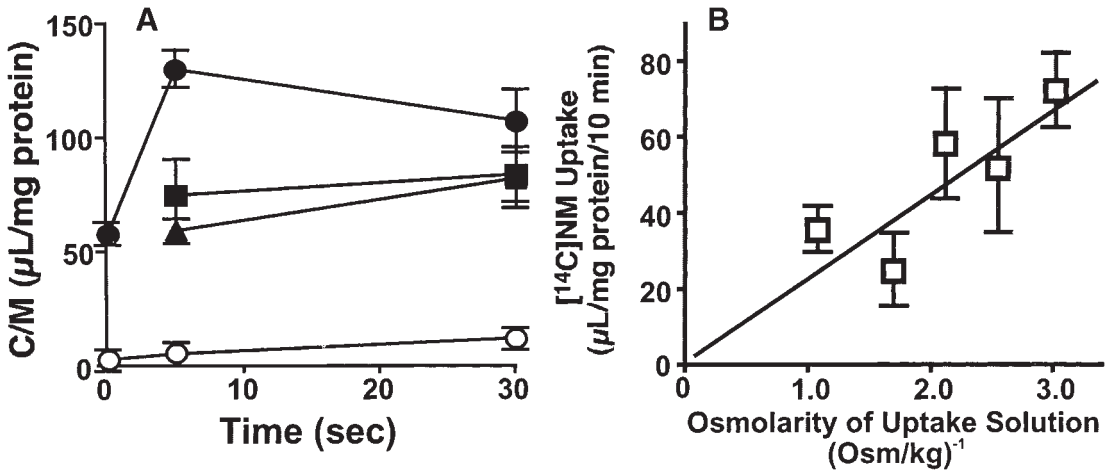

Figure 3. Uptake of NM by BBMVs isolated from rat renal cortex. (A) Uptake of [^{14}C]NM (0.7 μM) was measured with BBMVs (11.3 μg of protein) at 37°C (●), 25°C (■), and 4°C (▲). The uptake of [^{14}C]NM (0.7 μM) was also measured in the presence of unlabeled NM (1 mM) at 37°C (○). (B) The effect of osmolarity in the extravesicular medium on steady-state uptake (10 min) of [^{14}C]NM (1.4 μM) was measured in medium containing 150 mM NaCl and mannitol (20, 100, 200, 400, and 700 mM), at pH 7.4, that was buffered with 10 mM HEPES/Tris. Each point represents the mean ± SEM of four experiments.

the extravesicular medium on steady-state uptake of [^{14}C]NM by varying the concentration of mannitol in the outer medium. [^{14}C]NM uptake decreased linearly with a decrease of the reciprocal osmolarity (Fig. 3B). The surface binding of NM obtained from the extrapolation of osmolarity to infinity (y-intercept) was ~5% of that under the standard condition (isotonic condition). This result suggest that [^{14}C]NM is taken up into an intravesicular space and that the binding of NM to the membrane was negligible.

Involvement of Organic Cation Transporters in Apical Membrane of Proximal Tubule Epithelial Cells

We examined the inhibitory effect of NM on the uptake of typical substrates of hOCTN1 ([^{14}C]TEA) and hOCTN2 (L-[^{3}H]carnitine) by HEK293 cells stably expressing hOCTN1 and hOCTN2, respectively (Fig. 4). TEA (1 mM) and L-carnitine (100 μM) inhibited the uptake of the corresponding labeled compounds by HEK293 cells stably expressing hOCTN1 and hOCTN2, respectively. However, no inhibitory effect of NM was observed in HEK293 cells expressing hOCTN1 over the concentration range examined (0.5–500 μM, Fig. 4A), whereas an inhibitory effect of NM was observed only at 500 μM NM in HEK293 cells expressing hOCTN2 (Fig. 4B). No significant uptake of [^{14}C]NM was observed by HEK293 cells stably expressing hOCTN1 or hOCTN2 compared with mock cells (data not shown).

We also investigated whether NM is a substrate of P-gp. The permeability across cultured monolayers of LLC-GA5-COL150 cells was measured. The transport of [^{3}H]daunomycin, a typical substrate of P-gp, in the B-to-A direction was greater than that in the A-to-B transport (Fig. 5 inset). In contrast, no directional transcellular transport of NM was observed (Fig. 5). These results indicate that NM is not a substrate of hOCTN1, hOCTN2, or P-gp.

DISCUSSION

The physiological dichotomy of renal transport, including apparently discrete pathways that handle "organic cations" or "organic anions" has been a central tenant of renal physiology.[34,35] It has long been recognized that transcellular transport of both classes of compounds involves multiple parallel pathways.[36] NM is an organic cation that can be protonated at both the guanidine and amidino moieties. So it was suspected that NM is probably recognized as an organic cation. Because NM was reported to be concentrated in the kidney,[12] we investigated the

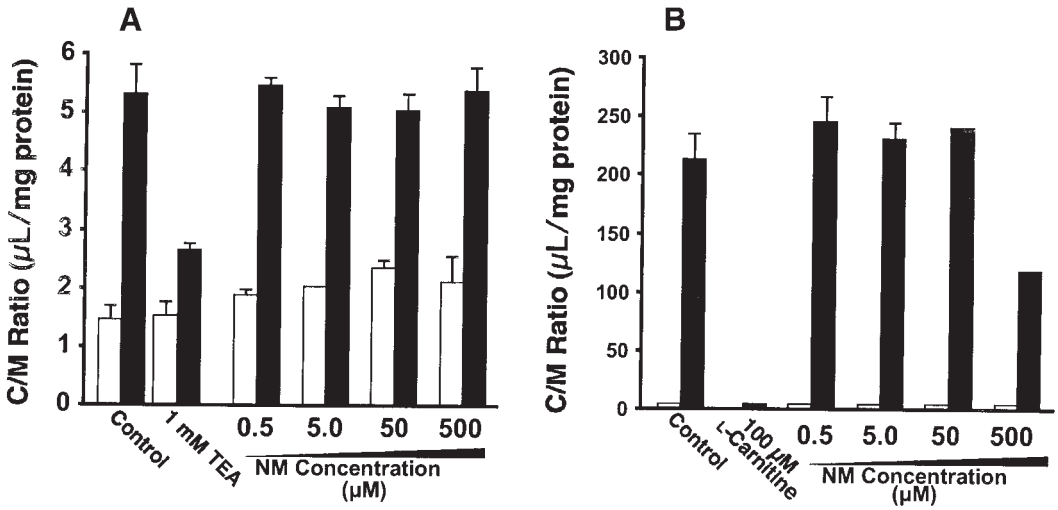

Figure 4. Inhibitory effect of NM on uptake of typical substrates of hOCTN1 and hOCTN2, respectively. The uptakes of (A) [^{14}C]TEA (6 μM) and (B) L-[^{3}H]carnitine (4.6 nM) were measured in the presence of various concentrations of unlabeled NM (from 0.5 to 500 μM) at 37°C, pH 7.4, for 3 min using HEK293 cells stably expressing hOCTN1 (A, closed), hOCTN2 (B, closed), and transfected with pcDNA3 alone (A, B, open), respectively. Each column represents the mean ± SEM of four experiments.

transport of NM across both the basolateral and brush-border membranes of proximal tubules, focusing on the involvement of an organic cation transport system(s).

First, NM transport across the basolateral membrane was investigated. We examined the initial uptake of [^{14}C]NM and [^{14}C]PAH *in vivo*.

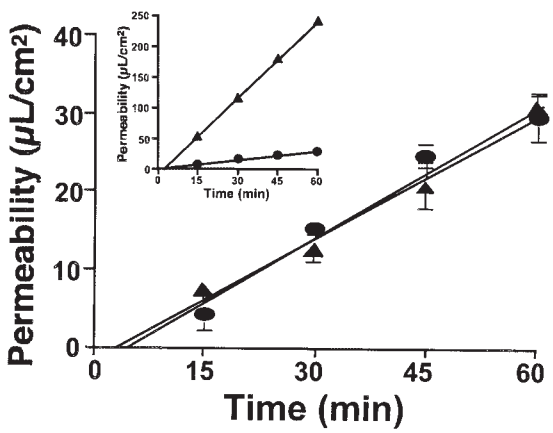

Figure 5. Transcellular transport of NM across cultured monolayers of LLC-GA5-COL150 cells. Permeability of NM (10 μM) was measured at 37°C for 60 min in HBSS ($pH_{apical}/pH_{basolateral} = 7.0:7.0$) across cultured monolayers of LLC-GA5-COL150 cells. That of [^{3}H]daunomycin (17.5 nM) was also measured (Fig. 5, inset). Circle and triangle symbols represent the transport in the apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions, respectively. Each column represents the mean ± SEM of three experiments.

PAH has been used as the prototypical substrate for the renal organic anion transporter (OAT),[36] and is extracted almost completely by the renal organic anion transport system during a single pass through the kidney. We found that the KUI of NM is close to that of PAH (Table 1), suggesting that NM is taken up into the renal cortex very quickly. TEA significantly blocked the renal uptake of NM, but did not affect that of PAH (Table 1), implying that an organic cation transport system is involved in NM uptake across the basolateral membrane of proximal tubules. We considered that the inhibitory effect of TEA (20 mM) on [^{14}C]NM uptake was low because the injected TEA was diluted rapidly. Using LLC-PK1 cells in suspension, we found that NM possibly blocked the uptake of [^{14}C]TEA in a competitive manner with high affinity, whereas the two metabolites of NM had little effect on [^{14}C]TEA uptake (Fig. 1C). In the basolateral membrane of proximal tubules, OCTs were reported to mediate transport of various organic cation substrates with high affinity; rOCT1 and rOCT2 were involved in rat kidney[16] and hOCT2 in human kidney.[17] We suggest that NM is a substrate of rOCT1, rOCT2, and hOCT2 (Fig. 2). Considering that transcellular experiments using LLC-PK1 cells revealed an efflux direction (B-to-A side) of NM transport (Fig. 1B), it seems likely that OCTs are involved in NM uptake across the basolateral membrane of proximal tubules.

Next, we investigated the transport of NM across brush-border membrane of proximal tubular epithelial cells. Unlabeled NM significantly decreased the uptake of [^{14}C]NM by BBMVs, whereas decrease of temperature reduced it only a little (Fig. 3A). Based on the results of NM uptake by BBMVs (Fig. 3A), it is implied that a transporter(s) is also involved in NM transport across the brush-border membrane. In the brush-border membrane of proximal tubules, OCTN1[18] and OCTN2[20] were shown to transport various organic cationic compounds, and P-gp also actively pumps out various hydrophobic xenobiotics to the lumen.[23] Human OCTN1 is a pH-dependent transporter for cationic compounds across the renal epithelial brush-border membrane, whereas hOCTN2 transports carnitine as well as cationic compounds. In this study, we found that NM is not a substrate of hOCTN1, hOCTN2, or P-gp. According to the results that NM is not excreted in the urine of human and rat, the reabsorption of NM should be efficient than glomerula filtration and tubular secretion of NM. Nevertheless, the uptake of NM by BBMVs depended on the proton gradient, and the transport of NM was not detected by OCTNs and P-gp (Figs. 4 and 5), suggesting that the contribution of hOCTNs and P-gp in brush-border membrane can be excluded. The mechanism for such the contradiction of results in rat BBMVs and hOCTNs remains poorly understood. NM is a substrate of OCTs that are localized in the basolateral membrane of proximal tubular cells but not of OCTNs or P-gp that localize in the brush-border membrane of proximal tubular cells. Therefore, the influx of NM from the basolateral membrane should result in the accumulation of NM in the kidney.

Some unknown transporter(s) may also be involved in the renal handing of NM transport. The plasma protein binding ratio of NM is ~40%, implying that NM could be excreted into urine partly through glomerular filtration. However, unchanged NM was not observed in the urine. Thus, NM is reabsorbed efficiently from the luminal side. Recently, OAT3 that is located in the basolateral membrane of proximal tubules[37] was reported to mediate transport of the cationic drug cimetidine.[38] In the apical membrane of proximal tubules, various transporters, such as NPT1,[39] and MRP1, MRP2, PEPT1, and PEPT2,[40] were reported to transport various drugs. Therefore, it is possible that other transporter(s) may also be involved in NM transport, in addition to the involvement of OCTs.

It is quite difficult to discuss the renal tubular secretion mechanism for NM in terms of a rate-limiting step involved in transcellular transport. NM is accumulated in the kidney, but not observed in the urine, implying that the uptake of NM from basolateral membrane and reabsorption of NM from apical membrane are efficient than the efflux and excretion, respectively. However, it is very difficult to separately determine the efficiency of each process. The specific inhibitor of the transporters involved in each process may be of help for us to clarify the rate-limiting step involved in transcellular transport of drugs.

In conclusion, we propose that the reason why NM accumulates in the kidney is that influx of NM at the basolateral membrane as a substrate of OCTs is highly efficient. Because the side effects of NM were suggested to be associated with the accumulation of NM, our finding here may provide a clue to a strategy for the prevention of the side effects of hyperkalemia and/or hyponatremia caused by NM in the future.

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