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Involvement of OCTN1 (*SLC22A4*) in pH-Dependent Transport of Organic Cations

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Abstract: OCTN1 (*SLC22A4*) transports cationic compounds such as tetraethylammonium in a pH-sensitive and sodium-independent manner in cultured cells, and is expressed in wide variety of tissues, including kidney, muscle, placenta, heart, and others. This study focused on the clarification of its subcellular distribution in kidney and on its driving force to throw light on the pharmacological and physiological roles of OCTN1. Uptake of [^{14}C]tetraethylammonium by membrane vesicles prepared from HEK293 cells stably transfected with human OCTN1 cDNA was osmolarity-sensitive, and the K_m of tetraethylammonium was 1.28 mM at intravesicular and extravesicular pH values of 6.0 and 7.4, respectively. Tetraethylammonium uptake was pH-dependent, and overshoot uptake was observed in the presence of an outwardly directed proton gradient. A protonophore and membrane potential affected the overshoot uptake. Furthermore, preloading tetraethylammonium in the vesicles significantly increased the rate of uptake of [^{14}C]tetraethylammonium. In mouse kidney, OCTN1 was expressed predominantly at the apical membrane of cortical proximal tubular epithelial cells. It was concluded that OCTN1 is involved in renal excretion of organic cations across the apical membrane in a pH-dependent, membrane potential-sensitive manner and is affected significantly by the organic cations on the trans side, showing counter transport activity.

Keywords: OCTN; organic cation; transporter; kidney; brush−border membrane; membrane transport; proton antiport

Introduction

Organic cations such as tetraethylammonium (TEA) are distributed into various tissues and excreted mainly into urine by specific membrane transport systems. Since renal epithelial transporters play a role in elimination of xenobiotics and endogenous metabolites into urine, clarification of their molecular mechanisms in kidney is important from pharmacological and physiological viewpoints. Previous studies have suggested that organic cations are excreted by a membrane potential-dependent transporter and by an organic cation/proton antiporter across the renal epithelial basolateral and apical membranes, respectively.[1−3] The organic cation transporters OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), and OCT3

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(1) Kinsella, J. L.; Holohan, P. D.; Pessha, N. I.; Ross, C. R. Transport of Organic Ions in Renal Cortical Luminal and Antiluminal Membrane Vesicles. *J. Pharmacol. Exp. Ther.* **1979**, *209*, 443−450.

(2) Takano, M.; Inui, K.; Okano, T.; Saito, H.; Hori, R. Carrier-mediated Transport Systems of Tetraethylammonium in Rat Renal Brush-border and Basolateral Membrane Vesicles. *Biochim. Biophys. Acta* **1984**, *773*, 113−124.

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(*SLC22A3*) have been isolated, and they exhibit membrane potential dependence.[4−10] Among them, OCT1 and OCT2 were suggested to be functional in kidney; subsequent studies on the localization of OCT1 and OCT2[11] and their functional characteristics clarified that OCT2 and OCT1 are mainly involved in the renal basolateral transport of organic cations, while no apically expressed organic cation transporters have been molecularly identified.[7−10] We have isolated and characterized a new family of organic cation transporters, OCTN. The first member of OCTN, OCTN1 (*SLC22A4*), was cloned from human fetal liver, and transports organic cations such as TEA in a pH-dependent manner.[12,13] The second member, OCTN2 (*SLC22A5*), transports physiologically important carnitine in an Na^+-dependent manner, as well as organic cations in an Na^+-independent manner.[14−18] OCTN2 is present in various tissues, including kidney, skeletal muscle, heart, placenta, and others,[14] and is expressed at the apical membrane of proximal tubular epithelial cells in kidney.[19] Both OCTN1 and OCTN2 transport carnitine and organic cations, though OCTN1 favors organic cations over carnitine and OCTN2 has the opposite preference.[20] Mouse OCTN2 transports carnitine and the organic cation TEA in Na^+-dependent and -independent manners, respectively.[20] Furthermore, hereditary mutant juvenile visceral steatosis mice that exhibit the phenotype of systemic carnitine deficiency, such as cardiac hypertrophy, lipid accumulation in the liver, and hyperammonemia[21,22] due to the functional loss of OCTN2 caused by a single amino acid mutation, Leu352Arg,[23,24] exhibited a significant alteration in the pharmacokinetics of TEA as well as carnitine, including changes in renal elimination.[25,26] Thus, it was strongly suggested that OCTN2 physiologically functions for re-

(3) Pritchard, J. B.; Miller, D. S. Mechanisms Mediating Renal Secretion of Organic Anions and Cations. *Physiol. Rev.* **1993**, *73*, 765−796.

(4) Gründemann, D.; Gorboulev, V.; Gambaryan, S.; Veyhl, M.; Koepsell, H. Drug Excretion Mediated by a New Prototype of Polyspecific Transporter. *Nature* **1994**, *372*, 549−552.

(5) Okuda, M.; Saito, H.; Urakami, Y.; Takano, M.; Inui, K. cDNA Cloning and Functional Expression of a Novel Rat Kidney Organic Cation Transporter, OCT2. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 500−507.

(6) Kekuda, R.; Prasad, P. D.; Wu, X.; Wang, H.; Fei, Y. J.; Leibach, F. H.; Ganapathy, V. Cloning and Functional Characterization of a Potential-sensitive, Polyspecific Organic Cation Transporter (OCT3) Most Abundantly Expressed in Placenta. *J. Biol. Chem.* **1998**, *273*, 15971−15979.

(7) Koepsell, H.; Gorboulev, V.; Arndt, P. Molecular Pharmacology of Organic Cation Transporters in Kidney. *J. Membr. Biol.* **1999**, *167*, 103−117.

(8) Zhang, L.; Brett, C. M.; Giacomini, K. M. Role of Organic Cation Transporters in Drug Absorption and Elimination. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 431−460.

(9) Sweet, D. H.; Pritchard, J. B. The Molecular Biology of Renal Organic Anion and Organic Cation Transporters. *Cell Biochem. Biophys.* **1999**, *31*, 89−118.

(10) Burckhardt, G.; Wolff, N. A. Structure of Renal Organic Anion and Cation Transporters. *Am. J. Physiol.* **2000**, *278*, F853−F866.

(11) Karbach, U.; Kricke, J.; Meier-Wentrup, F.; Gorboulev, V.; Volk, C.; Loffing-Cueni, D.; Kaissling, B.; Bachmann, S.; Koepsell, H. Localization of Organic Cation Transporters OCT1 and OCT2 in Rat Kidney. *Am. J. Physiol.* **2000**, *279*, F679−F687.

(12) Tamai, I.; Yabuuchi, H.; Nezu, J.; Sai, Y.; Oku, A.; Shimane, M.; Tsuji, A. Cloning and Characterization of a Novel Human pH-dependent Organic Cation Transporter, OCTN1. *FEBS Lett.* **1997**, *419*, 107−111.

(13) Yabuuchi, H.; Tamai, I.; Nezu, J.; Sakamoto, K.; Oku, A.; Shimane, M.; Sai, Y.; Tsuji, A. Novel Membrane Transporter OCTN1 Mediates Multispecific, Bidirectional, and pH-dependent Transport of Organic Cations. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 768−773.

(14) Tamai, I.; Ohashi, R.; Nezu, J.; Yabuuchi, H.; Oku, A.; Shimane, M.; Sai, Y.; Tsuji, A. Molecular and Functional Identification of Sodium Ion-dependent, High Affinity Human Carnitine Transporter OCTN2. *J. Biol. Chem.* **1998**, *273*, 20378−20382.

(15) Wu, X.; Prasad, P. D.; Leibach, F. H.; Ganapathy, V. cDNA Sequence, Transport Function, and Genomic Organization of Human OCTN2, a New Member of the Organic Cation Transporter Family. *Biochem. Biophys. Res. Commun.* **1998**, *246*, 589−595.

(16) Wu, X.; Huang, W.; Prasad, P. D.; Seth, P.; Rajan, D. P.; Leibach, F. H.; Chen, J.; Conway, S. J.; Ganapathy, V. Functional Characteristics and Tissue Distribution Pattern of Organic Cation Transporter 2 (OCTN2), an Organic Cation/Carnitine Transporter. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 1482−1492.

(17) Ohashi, R.; Tamai, I.; Yabuuchi, H.; Nezu, J.; Oku, A.; Sai, Y.; Shimane, M.; Tsuji, A. Na^+-dependent Carnitine Transport by Organic Cation Transporter (OCTN2): Its Pharmacological and Toxicological Relevance. *J. Pharmacol. Exp. Ther.* **1999**, *291*, 778−784.

(18) Wagner, C. A.; Lükewille, U.; Kaltenbach, S.; Moschen, I.; Bröer, A.; Risler, T.; Bröer, S.; Lang, F. Functional and Pharmacological Characterization of Human Na^+-Carnitine Cotransporter hOCTN2. *Am. J. Physiol.* **2000**, *279*, F584−F591.

(19) Tamai, I.; China, K.; Sai, Y.; Kobayashi, D.; Nezu, J.; Kawahara, E.; Tsuji, A. Na^+-coupled Transport of L-carnitine via High-affinity Carnitine Transporter OCTN2 and Its Subcellular Localization in Kidney. *Biochim. Biophys. Acta* **2001**, *1512*, 273−284.

(20) Tamai, I.; Ohashi, R.; Nezu, J.; Sai, Y.; Kobayashi, D.; Oku, A.; Shimane, M.; Tsuji, A. Molecular and Functional Characterization of Organic Cation/Carnitine Transporter Family in Mice. *J. Biol. Chem.* **2000**, *275*, 40064−40072.

(21) Koizumi, T.; Nikaido, H.; Hayakawa, J.; Nonomura, A.; Yoneda, T. Gene-dose Effect on Carnitine Transport Activity in Embryonic Fibroblasts of JVS Mice as a Model of Human Carnitine Transporter Deficiency. *Lab. Anim.* **1988**, *22*, 83−87.

(22) Horiuchi, M.; Yoshida, H.; Kobayashi, K.; Kuriwaki, K.; Yoshimine, K.; Tomomura, M.; Koizumi, T.; Nikaido, H.; Hayakawa, J.; Kuwajima, M.; Saheki, T. Cardiac Hypertrophy in Juvenile Visceral Steatosis (jvs) Mice with Systemic Carnitine Deficiency. *FEBS Lett.* **1993**, *326*, 267−271.

(23) Hashimoto, N.; Suzuki, F.; Tamai, I.; Nikaido, H.; Kuwajima, M.; Hayakawa, J.; Tsuji, A. Gene-dose Effect on Carnitine Transport Activity in Embryonic Fibroblasts of jvs Mice as a Model of Human Carnitine Transporter Deficiency. *Biochem. Pharmacol.* **1998**, *55*, 1729−1732.

(24) Nezu, J.; Tamai, I.; Oku, A.; Ohashi, R.; Yabuuchi, H.; Hashimoto, N.; Nikaido, H.; Sai, Y.; Koizumi, A.; Shoji, Y.; Takada, G.; Matsuishi, T.; Yoshino, M.; Kato, H.; Ohura, T.; Tsujimoto, G.; Hayakawa, J.; Shimane, M.; Tsuji, A. Primary Systemic Carnitine Deficiency is Caused by Mutations in a Gene Encoding Sodium Ion-dependent Carnitine Transporter. *Nat. Genet.* **1999**, *21*, 91−94.

absorption of carnitine filtered through glomeruli, and at the same time, it partially contributes to secretion of organic cations across the epithelial apical membrane.[26] However, OCTN2 might not be a major organic cation transporter in the apical membrane.[27] In addition, there is a possibility that the renal apical membrane includes multiple transporter molecules,[28] and OCTN1 is a better transporter for organic cations than OCTN2. Furthermore, OCTN1 shows pH sensitivity in TEA transport,[12,13] and its amino acid sequence is 31 and 33% similar to those of OCT1 and OCT2, respectively. These previous observations prompted us to examine whether OCTN1 is involved in renal secretion of organic cations across the apical membranes of proximal tubular epithelial cells.

The purpose of this study was to examine the subcellular distribution of OCTN1 in kidney and to identify the driving force for the OCTN1-mediated transport of organic cations to establish whether OCTN1 is involved as a renal epithelial apical membrane transporter for organic cations. Utilization of membrane vesicles can provide direct evidence for the nature of the energy that drives transport of organic cations via OCTN1, and therefore, we used membrane vesicles prepared from cultured cells stably transfected with human OCTN1 cDNA for this study.

Experimental Section

Chemicals. L-[*ethyl*-^{14}C]Tetraethylammonium bromide (55 mCi/mmol) was purchased from Moravec Biochemicals Inc. (Brea, CA). Other reagents, including those for cell culture, membrane preparation, transport experiments, and immunostainings, were of the highest grade available. HEK293 cells were obtained from Health Science Research Resources Bank (Tokyo, Japan).

Cell Culture and Cellular Transport Study. HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37 °C and 5% CO_2. HEK293 cells were transfected with human OCTN1 cDNA subcloned into the *Eco*RI site of the pcDNA3 or pcDNA3 vector alone by using LipofectAMINE as described previously.[14,19] The cells were harvested and suspended in buffer 1 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, adjusted to pH 7.4 with NaOH. The cell suspension and a solution containing the radiolabeled TEA in the transport medium were separately incubated at 37 °C for 20 min, and then the transport experiment was initiated by mixing them. At an appropriate time after initiation of the transport reaction, 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 silicone oil (SH550, Toray Dow Corning Co., Tokyo, Japan) and liquid paraffin (Wako Pure Chemical Industries) with a density of 1.03 onto a 3 N KOH solution placed under the silicon oil−paraffin layer. After solubilization of each cell pellet in 3 N KOH, the cell lysate was neutralized with HCl. Then, the associated radioactivity was measured by means of a liquid scintillation counter using Cleasol-1 as a liquid scintillation fluid (Nacalai tesque, Kyoto, Japan).

Preparation of Plasma Membrane Vesicles and Transport Study. The membrane vesicles were prepared essentially as described previously.[19] Briefly, the obtained cell line that expresses human OCTN1 was cultured in the presence of 1 mg/mL G418. The confluent cells were harvested, washed, suspended in 25 mL of buffer 2 [10 mM NaCl, 1.5 mM $MgCl_2$, 0.02 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM Tris-HCl (pH 7.4)], and placed on ice for 30 min. The cells were disrupted using a nitrogen bomb (Parr 4635) at 700 psi, homogenized, and separated by sucrose gradient centrifugation, and membrane vesicles were finally obtained as a pellet after centrifugation at 100000*g* for 3 h. The final pellet was suspended usually in buffer 3 [100 mM KCl, 100 mM mannitol, 0.02 mM PMSF, and 20 mM Mes/Tris (pH 6.0)] and stored at −80 °C until it was used. The content of protein in each preparation was measured by using a Bio-Rad protein assay kit.[29]

Transport studies were performed by a rapid filtration technique at 25 °C. Membrane vesicles stored at −80 °C were thawed rapidly at 37 °C and kept on ice. After the vesicles had been preincubated for 30 min at 25 °C, 10 μL of the membrane vesicle suspension containing 20 μg of protein was mixed with 90 μL of a drug solution containing the test compound in buffer 4 [usually 100 mM KCl, 100 mM mannitol, and 20 mM Hepes/Tris (pH 7.4)] to initiate uptake studies. The uptake reaction was terminated by diluting the mixture with 1 mL of buffer 5 [100 mM KCl, 100 mM mannitol, 5 mM TEA, and 20 mM Hepes/Tris (pH 7.4)] at 4 °C, and the mixture was immediately filtered through a membrane filter (Millipore, HAWP, 0.45 μm pore size) that had been presoaked with a 5 mM TEA solution overnight, and washed four times with buffer 5. The radioactivity retained on the membrane filter was quantitated with a liquid scintillation counter. The background radioactivity was evaluated by mixing an ice-cold [^{14}C]TEA solution with membrane vesicles that had previously been

(25) Yokogawa, K.; Higashi, Y.; Tamai, I.; Nomura, M.; Hashimoto, N.; Nikaido, H.; Hayakawa, J.; Miyamoto, K.; Tsuji, A. Decreased Tissue Distribution of L-carnitine in Juvenile Visceral Steatosis Mice. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 224−230.

(26) Ohashi, R.; Tamai, I.; Nezu, J.; Nikaido, H.; Hashimoto, N.; Oku, A.; Sai, Y.; Shimane, M.; Tsuji, A. Molecular and Physiological Evidence for Multifunctionality of Carnitine/Organic Cation Transporter OCTN2. *Mol. Pharmacol.* **2001**, *59*, 358−366.

(27) Chun, J. K.; Zhang, L.; Piquette-Miller, M.; Lau, E.; Tong, L. Q.; Giacomini, K. M. Characterization of Guanidine Transport in Human Renal Brush Border Membranes. *Pharm. Res.* **1997**, *14*, 936−941.

(28) Ohnishi, S.; Saito, H.; Fukada, A.; Inui, K. Independent Organic Cation Transport Activity of Na^+-L-Carnitine Cotransport System in LLC-PK(1) Cells. *Am. J. Physiol.* **2001**, *281*, F273−F279.

(29) Bradford, M. M. A. Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248−254.

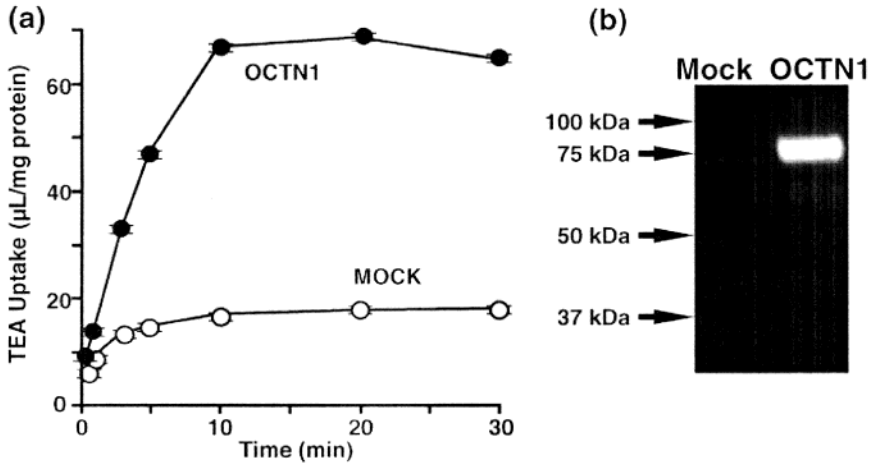

Figure 1. Establishment of a stably OCTN1-expressing HEK293 cell line. (a) The uptake of [^{14}C]TEA (20 μM) by OCTN1-expressing cells (●) and mock cells (○) was measured at 37 °C by the silicone layer centrifugation method at pH 7.4. The results are shown as means ± the standard error of the mean (SEM) of four determinations. (b) Expression of OCTN1 protein was detected by Western blot analysis using anti-human OCTN1 antibody (30-fold dilution), and 5 μg of protein was applied to each lane. Molecular sizes are shown with arrows in kilodaltons, and the specific band was obtained only in OCTN1-transfected HEK293 cells.

diluted with ice-cold buffer 5, followed immediately by filtration. Uptake was usually shown as permeability (microliters per milligram of protein) obtained by dividing the uptake amount by the extravesicular concentration of TEA.

Immunohistochemical Study of OCTN1 in Kidney and Western Blot Analysis. Rabbit polyclonal antibodies for mouse OCTN1 and mouse OCTN2 and frozen sections of kidney were prepared as described previously.[19,20] The sections were first heated to 92 °C in the RETRIEVE-ALL (SIGNET Pathology Systems Inc., Dedham, MA) buffer. They were then incubated with a mixture of anti-OCTN antibody and anti-Na^+/K^+ ATPase mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) for 1 h, and subsequently incubated with secondary antibodies (Alexa Fluoro 594 goat anti-rabbit IgG conjugate and Alexa Fluor 488 goat anti-mouse IgG conjugate) for 30 min at room temperature. Finally, they were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to fix the sample and to stain nuclei. The specimens were examined with an Axiovert S 100 microscope (Carl Zeiss, Jena, Germany), and the images were captured with an AxioCam (Carl Zeiss). For Western blot analysis, the rabbit polyclonal antibody was raised against a synthesized polypeptide near the carboxyl terminus of human OCTN1 with an amino acid sequence of WFRSGKKTRDSMETEENPK. HEK293 cells stably expressing human OCTN1 or mock cells were grown to confluence and harvested. The sample treatment and detection for Western blot analysis were essentially as described previously.[20]

Results

Expression of OCTN1 Protein and Transport Activity of [^{14}C]TEA in HEK293 Cells Stably Transfected with Human OCTN1 cDNA. Among HEK293 cell lines transfected with human OCTN1 cDNA, one clone was selected as a stably OCTN1-expressing cell line. Figure 1a shows the uptake of [^{14}C]TEA by the cells compared with the uptake by cells transfected with the expression vector pcDNA3 alone (mock). Uptake of [^{14}C]TEA by the OCTN1-transfected cell line was very rapid, and steady-state uptake corresponded to ~10-fold accumulation over the outer medium concentration when calculated using a cell volume of 6.3 μL/mg of protein.[12] In contrast, mock cells showed little accumulation and the initial uptake rate was slow. These results are consistent with those obtained with transiently transfected cells.[12,13] Figure 1b shows an expression of human OCTN1 protein in HEK293 cells that were transfected with OCTN1 cDNA, while its expression in mock cells was undetectable. The molecular size of expressed OCTN1 protein was ~75 kDa and was slightly larger than the estimated molecular size from amino acid sequence (62 173 Da), suggesting the modification of the expressed protein by glycosylation. These results confirmed that the cell line was suitable for further characterization of OCTN1 function, if apparent uptake by the OCTN1-expressing cells is corrected for the uptake by mock cells.

Osmolarity Effect on TEA Uptake by Membrane Vesicles. Uptake of [^{14}C]TEA by membrane vesicles prepared from HEK293 cells that were transfected with human OCTN1 cDNA (OCTN1 vesicles) or expression vector pcDNA3 alone (mock vesicles) was measured at various levels of osmolarity in uptake or washing medium. Figure 2a shows the initial uptake of [^{14}C]TEA by OCTN1 and mock vesicles using isotonic or hypotonic washing medium. The apparent uptake in hypotonic washing medium represents the binding to the membrane because hypotonic washing medium collapses the membrane vesicles and intravesicular uptake accounts for the difference in uptake between the two washing media. In OCTN1 vesicles, a significant decrease in the uptake of [^{14}C]TEA was observed

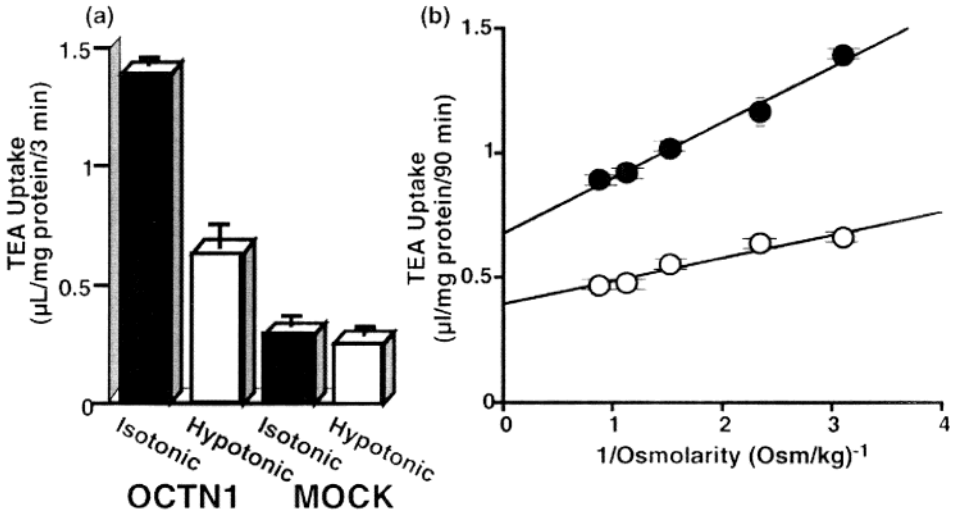

Figure 2. Osmolarity effect on [^{14}C]TEA uptake at the initial (a) and steady state (b). (a) Uptake of [^{14}C]TEA (36 μM) by OCTN and mock vesicles was measured using isotonic [100 mM NaCl, 100 mM mannitol, 20 mM HEPES/Tris, and 5 mM TEA (pH 7.4), 321 mOsm/kg, (■)] or hypotonic washing medium [10 mM HEPES/Tris and 5 mM TEA (pH 7.4), 20 mOsm/kg (□)] at 3 min. (b) Steady-state uptake of [^{14}C]TEA (36 μM) by OCTN1 vesicles (●) and mock vesicles (○) was measured at 90 min at various values of osmolarity in the uptake medium [100, 200, 400, 600, or 800 mM mannitol, 100 mM KCl, and 20 mM HEPES/Tris (pH 7.4)] from 320 to 1150 mOsm/kg. The solid line was obtained by linear regression. The results are shown as means ± the standard error of the mean of four determinations.

when hypotonic medium was used, while no change was observed in the case of mock vesicles. Accordingly, transport into the vesicles, and not binding, should largely account for the apparent initial uptake by OCTN1 vesicles under standard isotonic conditions. Figure 2b shows the steady-state uptake of [^{14}C]TEA by OCTN1 and mock vesicles measured at 90 min using uptake medium with increasing osmolarity from 320 to 1150 mOsm/kg. Uptake of [^{14}C]TEA linearly decreased with the increase in osmolarity in both vesicles. The extrapolated ordinate intercept may correspond to the membrane binding of [^{14}C]TEA. In OCTN1 vesicles, the uptake was more osmolarity-sensitive than that by mock vesicles and the estimated level of binding to OCTN1 vesicles might be higher than that to mock vesicles. Thus, although binding cannot be completely neglected, the osmolarity effect on both the steady-state and initial uptake of [^{14}C]TEA demonstrated that, by taking the difference in the uptake by OCTN1 and mock vesicles, it is possible to evaluate adequately the OCTN1-mediated transport of TEA.

Effect of pH on TEA Transport by Membrane Vesicles. The time course of the uptake of [^{14}C]TEA by membrane vesicles was examined at pH_{in} and pH_{out} values of 6.0 and 6.0, or 6.0 and 7.4, respectively. As shown in Figure 3a, at pH_{in} 6.0 and pH_{out} 6.0 or 7.4, uptake of [^{14}C]TEA by OCTN1 vesicles was significantly higher than that by mock vesicles. In the presence of an outwardly directed proton gradient (pH_{out} 7.4), initial uptake was greater than that at pH_{out} 6.0 and showed overshoot uptake, suggesting that the outwardly directed proton gradient drove the uptake of TEA by OCTN1. Figure 3b shows the effect of external pH from 6.0 to 8.0 on the initial uptake of [^{14}C]TEA at pH_{in} 6.0. In OCTN1 vesicles, a significant influence of pH_{out} on the initial uptake of [^{14}C]TEA was observed with maximum uptake at pH_{out} 7.0, while such a change was not observed in mock vesicles. The specific uptake by OCTN1, obtained as the difference between uptake by OCTN1 and mock vesicles, was maximal at neutral pH. The observation of the maximum uptake well corresponded to the previous report that TEA uptake by rat brush−border membrane vesicles was optimal at neutral pH and was explained by the presence of the optimal form of protonation and deprotonation of the functional sites of the H^+/organic cation antiport system.[30] The effect of pH on the efflux of [^{14}C]TEA was also studied at pH_{in} 7.4, with an increase in pH_{out} from 5.5 to 8.0. As shown in Figure 3c, at acidic pH_{out}, the efflux rate was greater than at higher pH. Thus, the transport mediated by OCTN1 is dependent on pH, and TEA may be transported via a proton antiport mechanism, since the rate of TEA transport was high in the presence of a proton gradient.

Concentration Dependence of TEA Uptake by Membrane Vesicles. The concentration dependence of TEA uptake by OCTN1 was studied at pH_{in} and pH_{out} values of 6.0 and 7.4, respectively. The result, obtained as the difference in uptake between OCTN1 and mock vesicles, is shown in Figure 4a. The Eadie−Hofstee plot (Figure 4b) exhibited a single straight line, and the kinetic parameters V_{max} and K_m were 1.96 ± 0.25 pmol (2 min)$^{-1}$ (mg of protein)$^{-1}$ and 1.28 ± 0.22 mM, respectively. The results demonstrate that OCTN1 has a single functional site for TEA transport and the affinity is close to that obtained in HEK293 cells (K_m = 0.44 mM) transiently transfected with OCTN1 cDNA.[12]

(30) Maegawa, H.; Kato, M.; Inui, K.; Hori, R. pH Sensitivity of H^+/Organic Cation Antiport System in Rat Renal Brush-border Membranes. *J. Biol. Chem.* **1988**, *263*, 11150−11154.

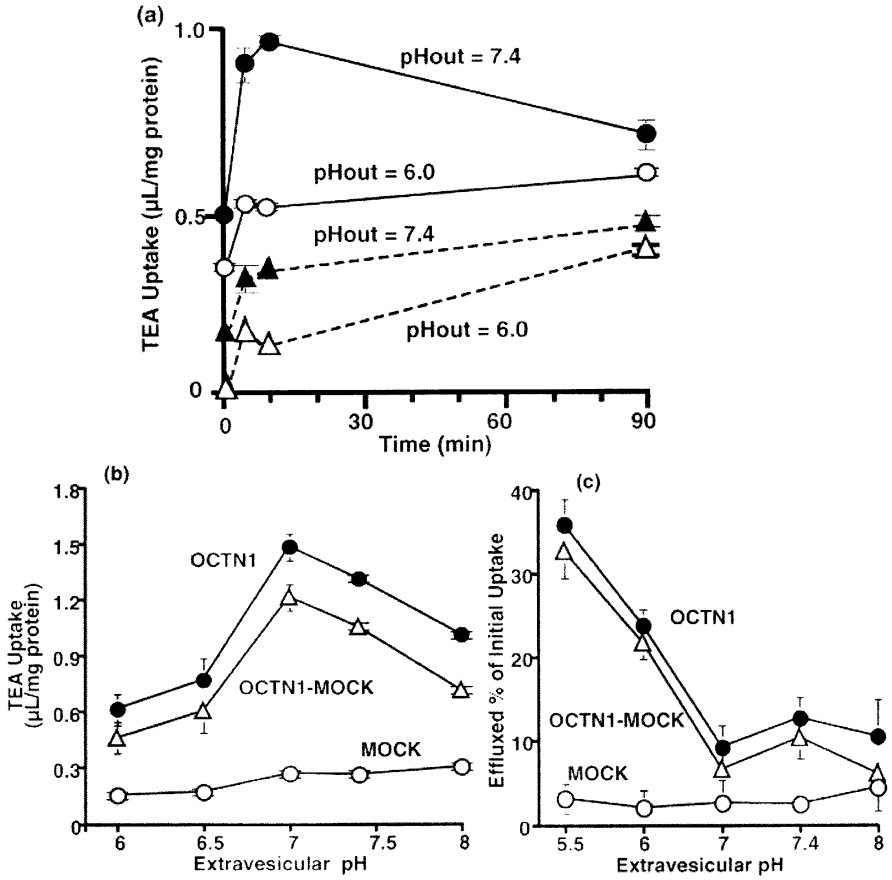

Figure 3. Effect of pH on uptake (a and b) and efflux (c) of [^{14}C]TEA. (a) The time course of the uptake of [^{14}C]TEA was measured at pH_{in} 6.0 and pH_{out} 6.0 (○ and △) or 7.4 (● and ▲) for OCTN1 vesicles (● and ○) and mock vesicles (▲ and △). (b) Uptake of [^{14}C]TEA was measured at pH_{in} 6.0 and variable pH_{out} values from 6.0 to 8.0 for 2 min by OCTN1 vesicles (●) and mock vesicles (○). OCTN1-mediated uptake, obtained by subtracting the uptake by mock vesicles from that by OCTN1 vesicles, is also shown (△). (c) Efflux of [^{14}C]TEA was measured at various pH_{out} values after preloading of 180 [OCTN1 vesicles (●)] and 270 μM [^{14}C]TEA [mock vesicles (○)] into vesicles at pH 7.4 for 60 min. The effluxed amount of [^{14}C]TEA was evaluated from the remaining amount of [^{14}C]TEA in membrane vesicles at 10 s and is shown as a percentage of the initial amount of [^{14}C]TEA in the vesicles. OCTN1-mediated efflux, obtained by subtracting the amount effluxed in mock vesicles from that in OCTN1 vesicles, is also shown (△). The results are shown as means ± the standard error of the mean of four determinations.

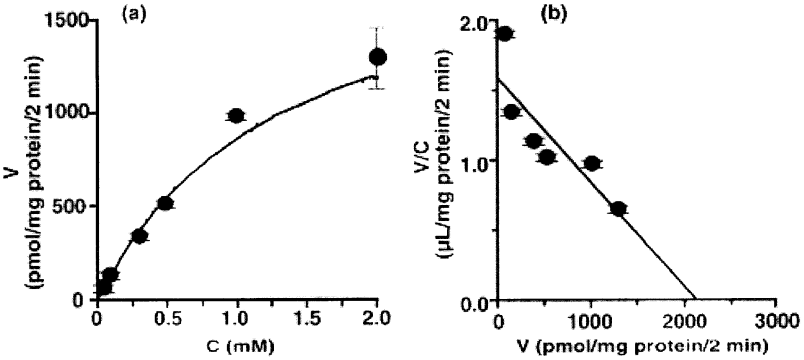

Figure 4. Concentration dependence of TEA uptake. (a) Initial uptake of TEA by membrane vesicles at various concentrations of TEA from 36 μM to 2 mM was measured for 2 min at pH_{in} 6.0 and pH_{out} 7.4. The result shows OCTN1-mediated uptake after subtracting uptake by mock vesicles from that of OCTN1 vesicles (a). An Eadie−Hofstee plot of the result is also shown (b). The solid line represents the uptake estimated from the kinetic parameters, K_m and V_{max}, shown in Results. The results are means ± the standard error of the mean of four determinations.

Effects of Proton Gradient, Membrane Potential, and Trans Stimulation on TEA Uptake. To clarify the underlying mechanism of the apparent pH dependence of TEA transport via OCTN1, the effect of a pH gradient on the overshoot uptake of [^{14}C]TEA was further examined by using a protonophore, carbonyl cyanide *p*-trifluoromethoxyphen-

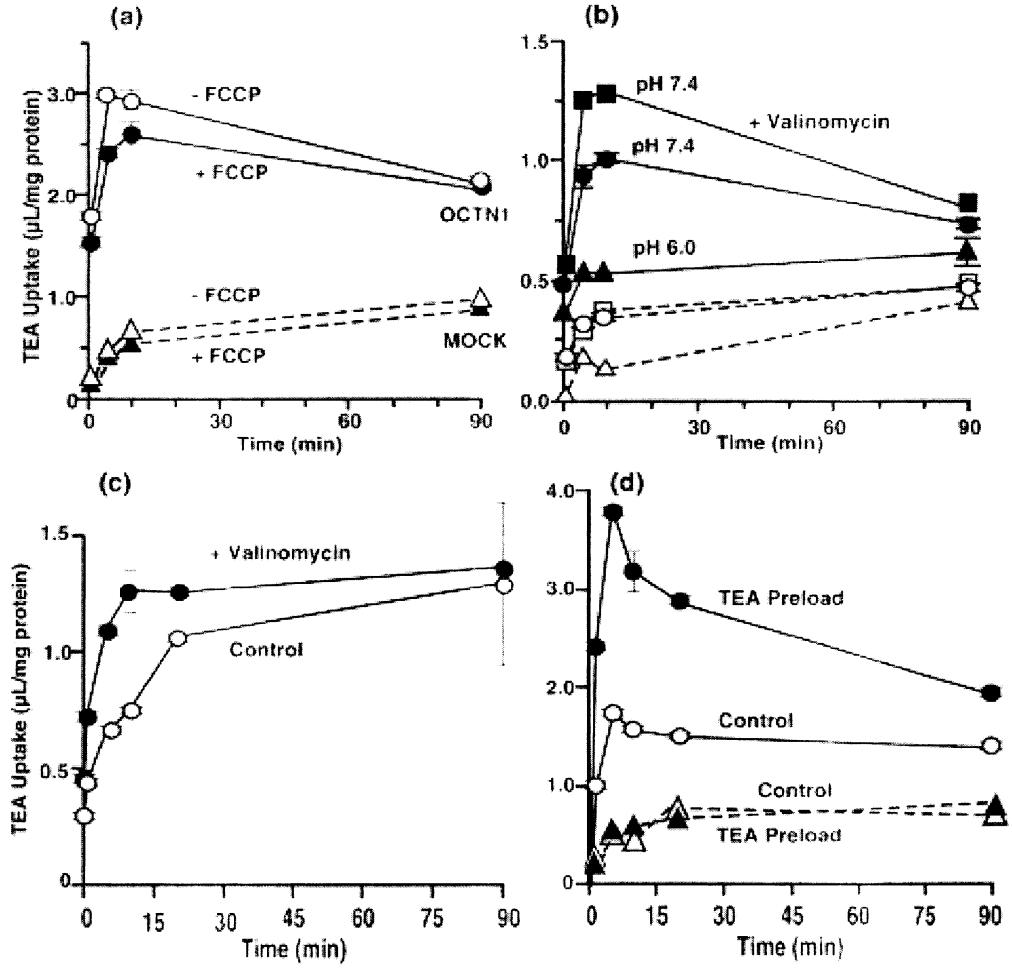

Figure 5. Effect of proton gradient, membrane potential, and trans stimulation on [^{14}C]TEA uptake. Uptake of [^{14}C]TEA was measured by preloading membrane vesicles with buffer 3, and uptake was measured using buffer 5 [10 mM KCl, 90 mM *N*-methylglucamine, 100 mM mannitol, and 20 mM Hepes/Tris (pH 7.4)] in the presence (● and ▲) or absence (○ and △) of 50 μM FCCP by OCTN1 vesicles (● and ○) and mock vesicles (▲ and △). FCCP and valinomycin (10 μM) were preincubated with membrane vesicles for 30 min immediately before the experiment to initiate uptake and were also included in the uptake buffer. (b) Uptake of [^{14}C]TEA was measured by preloading membrane vesicles with buffer 3, and uptake was measured using buffer 5 in the presence (■ and □) or absence (●, ○, ▲, and △) of 10 μM valinomycin by OCTN1 vesicles (■, ●, and ▲) and mock vesicles (□, ○, and △). Uptake of [^{14}C]TEA was also studied in the absence of a pH gradient at pH_{out} 6.0 (▲ and △) without valinomycin. Valinomycin was preincubated with membrane vesicles for 30 min immediately before the experiment to initiate uptake and was also used during the uptake experiment. (c) Uptake of [^{14}C]TEA was measured in the same manner described above (panel b) at pH_{in} 6.0 and pH_{out} 6.0 in the presence (●) or absence (○) of valinomycin in OCTN1 vesicles. (d) Uptake of [^{14}C]TEA was measured by preloading membrane vesicles with buffer 2 containing 2 mM TEA for 30 min, and the uptake was measured using buffer 4. The results with OCTN1 vesicles (● and ○) and mock vesicles (▲ and △) with (● and ▲) or without preloading TEA (○ and △) are shown. Each value represents the mean ± the standard error of the mean of three determinations.

ylhydrazone (FCCP). As shown in Figure 5a, in the presence of FCCP (pH_{in} 6.0 and pH_{out} 7.4), the overshoot uptake of TEA by OCTN1 was reduced without a change in the uptake at the steady state. In this study, valinomycin was included to maintain membrane potential, which might be altered due to proton flux induced by FCCP. The decrease in overshoot uptake by FCCP and the above-described pH effects strongly indicate that OCTN1 functions as a proton/organic cation antiporter. Figure 5b shows the effect of membrane potential on the TEA transport via OCTN1. In this study, an outwardly directed K^+ gradient was imposed and the uptake of [^{14}C]-TEA was evaluated with or without valinomycin at pH_{in} 6.0 and pH_{out} 7.4. By imposing an inside negative membrane potential with valinomycin, we further increased the overshoot uptake driven by the pH gradient without a significant change in the steady-state uptake. Such an increase in the uptake was not observed in mock vesicles. Accordingly, the pH gradient-dependent transport is suggested to be electrogenic. When we studied the effect of membrane potential in the absence of a proton gradient (pH_{in} 6.0 and pH_{out} 6.0), a small increase in TEA uptake but no overshoot uptake was seen with inside-negative membrane potential (Figure 5c). Accordingly, OCTN1-mediated transport of TEA is affected by both pH and membrane potential; however, uphill

transport of TEA may predominantly depend on the proton gradient, and membrane potential may simply facilitate the proton gradient-dependent transport.

Since transport of organic cations in renal apical membrane vesicles often exhibits a trans stimulation effect,[31] the effect of 2 mM preloaded TEA in vesicles on [^{14}C]TEA uptake was examined. As shown in Figure 5d, preloading of unlabeled TEA significantly increased the uphill uptake of [^{14}C]TEA by OCTN1 vesicles at pH_{in} 6.0 and pH_{out} 7.4, while such an increase was not observed in mock vesicles. The apparent increment in the initial uphill uptake of [^{14}C]TEA was significantly higher than that generated by a proton gradient or membrane potential, and cationic compounds on the trans side may be the preferred driving force for OCTN1-mediated TEA transport.

Immunohistochemical Detection of OCTN1 in Mouse Kidney. Since OCTN1 was strongly expressed in kidney,[12,20,32] the subcellular localization of OCTN1 in mouse kidney was examined by using anti-mouse OCTN1 antibody. Panels a and b of Figure 6 show the cortical localization of OCTN1 and OCTN2 proteins in mouse kidney, respectively. Here, OCTN2 and Na^+/K^+ ATPase were used as markers of apical and basolateral membranes, respectively.[19,33] Both OCTN1 and OCTN2 proteins are shown in red, and green shows Na^+/K^+ ATPase, which was expressed in the cell membrane through the whole tissue, including cortex and medulla, and at an especially high level in distal tubules and thick ascending limb of Henle.[33] Although OCTN2 is known to be localized at the apical membrane of renal proximal tubular epithelial cells,[19] its limited distribution in cortex and negligible distribution in medulla were clarified for the first time in this study (Figure 6b,d). OCTN1 was localized in cortex in a manner similar to that of OCTN2 (Figure 6a,c), since the region that corresponds to medulla according to staining of Na^+/K^+ ATPase was not stained.[33] Panels c and d of Figure 6 show the subcellular distributions of OCTN1 and OCTN2, respectively, in mouse kidney (shown in red; green and blue show Na^+/K^+ ATPase and nuclei, respectively). OCTN1 and OCTN2 were both expressed in regions distinct from Na^+/K^+ ATPase, showing the localization in the apical membrane of proximal tubular epithelial cells. They were not found in glomeruli. Accordingly, OCTN1 is localized in the apical membrane of renal proximal tubular epithelial cells in the same manner as OCTN2, and their localization is distinct from that of OCT transporters.

Discussion

Previously identified cation transporters OCT1 and OCT2 are characterized by localization in the basolateral membrane of renal tubular epithelial cells, and molecular identification of apical membrane cation transporters remains to be achieved. We and Wu et al. reported that OCTN1 transports organic cations such as TEA, pyrilamine, and quinidine in an Na^+-independent but pH-dependent manner when expressed in cultured cells.[12,13,32] Furthermore, since it was previously suggested that organic cation transport at the apical membrane is pH-dependent, but not affected by membrane potential,[1−3,7−10] it was expected that OCTN1 might be involved as a pH-dependent organic cation transporter at the renal apical membrane. Accordingly, subcellular localization in the kidney and driving force, which will be key features in identifying the role of OCTN1 as one of cation transporters, were examined in this study. Since driving force can be directly evaluated by means of a membrane vesicle study, we prepared membrane vesicles from cells stably transfected with human OCTN1 for use in this study.

As is clearly shown in Figure 6, both OCTN1 and OCTN2 were expressed in the apical membrane of renal proximal tubular epithelial cells, but not at the basolateral membrane. Furthermore, all the results obtained in these studies demonstrated that OCTN1 was able to transport typical organic cation TEA depending on pH gradient as well as pH and membrane potential. Accordingly, OCTN1 may be a novel type of cation transporter driven by both proton gradient and membrane potential. However, as shown in Figure 5c, uphill transport was not so significant upon induction of an inside-negative membrane potential at pH_{in} 6.0 and pH_{out} 6.0, while electrogenic uphill transport was observed in the presence of an outwardly directed proton gradient (pH_{in} 6.0 and pH_{out} 7.4), as shown in Figure 5b. Accordingly, for uphill transport, a proton gradient may be primarily important and membrane potential alone may not be able to cause uphill transport, but may only accelerate the pH-dependent uphill transport of TEA.

This study also showed an extensive uphill transport of [^{14}C]TEA after preloading unlabeled TEA, indicating countertransport (Figure 5d). Organic cation/organic cation antiport can presumably account for this observation, and it suggested that OCTN1 was more active as an organic cation/organic cation antiporter than as a proton/organic cation antiporter. Similar trans stimulated uptake was observed in human kidney apical membrane vesicles[34] as well as in animals,[31,35] so organic cation/organic cation antiport via OCTN1 is also a possible mechanism for physiological uphill

(31) Holohan, P. D.; Ross, C. R. Mechanisms of Organic Cation Transport in Kidney Plasma Membrane Vesicles: 1. Countertransport Studies. *J. Pharmacol. Exp. Ther.* **1980**, *215*, 191−197.

(32) Wu, X.; George, R. L.; Huang, W.; Wang, H.; Conway, S. J.; Leibach, F. H.; Ganapathy, V. Structural and Functional Characteristics and Tissue Distribution Pattern of Rat OCTN1, an Organic Cation Transporter, Cloned from Placenta. *Biochim. Biophys. Acta* **2000**, *1466*, 315−327.

(33) Siegel, G. J.; Holm, C.; Schreiber, J. H.; Desmond, T.; Ernst, S. A. Purification of Mouse Brain (Na^+K^+)-ATPase Catalytic Unit, Characterization of Antiserum, and Immunocytochemical Localization in Cerebellum, Choroid plexus, and Kidney. *J. Histochem. Cytochem.* **1984**, *32*, 1309−1318.

(34) Ott, R. J.; Hui, A. C.; Yuan, G.; Giacomini, K. M. Organic Cation Transport in Human Renal Brush-border Membrane Vesicles. *Am. J. Physiol.* **1991**, *261*, F443−F451.

(35) Katsura, T.; Maegawa, H.; Tomita, Y.; Takano, M.; Inui, K.; Hori, R. Trans-stimulation Effect on H^+-Organic Cation Antiport System in Rat Renal Brush-border Membranes. *Am. J. Physiol.* **1991**, *261*, F774−F778.

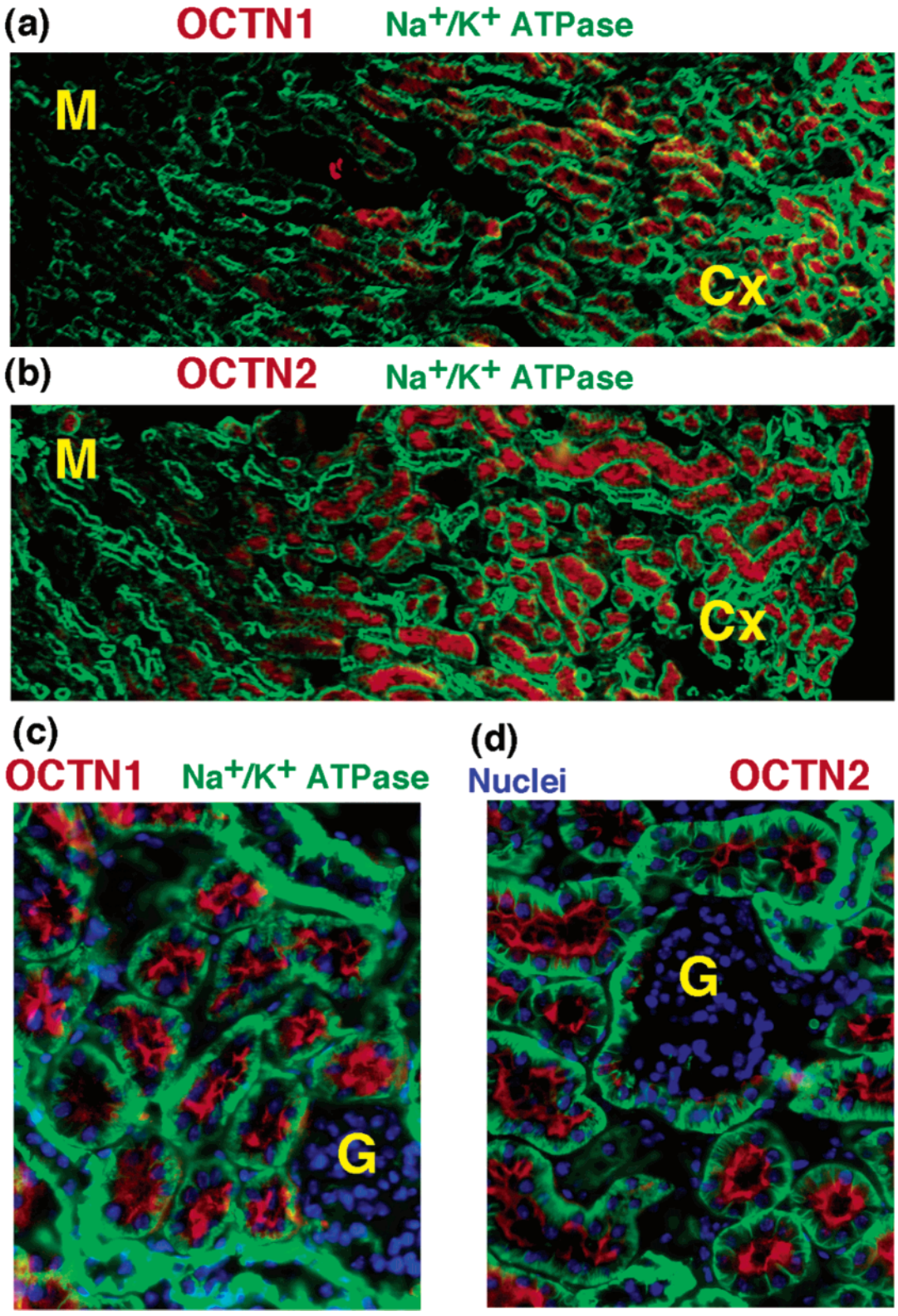

Figure 6. Immunolocalization of OCTN1 (a and c) and OCTN2 (b and d) proteins in kidney of mice. Cryosections (10 μm) of mouse kidney were incubated with the affinity-purified antiserum (20-fold dilution) against mouse OCTN1 (a and c) or mouse OCTN2 (b and d) and examined under an Axiovert S 100 microscope. Expression of OCTN1 (a) and OCTN2 (b), shown in red, was observed at tubular epithelial cells in the cortex (Cx) and was negligible in the medulla (M). OCTN1 (c) and OCTN2 (d) were localized at the apical membrane of the proximal tubular epithelial cells as shown in red, but not in glomeruli (G). Green and blue represent staining of Na^+/K^+ ATPase and nuclei, respectively.

transport. However, the countertransported compounds present in tubular lumen for the renal excretion of organic cations are not clear at present. In human kidney membrane vesicles, uptake of *N*-methylnicotinamide (NMN) did not show trans stimulation by other cations, and our previous study did not show an inhibitory effect of NMN on TEA uptake, though two studies observed strong inhibition by quinidine.[13,34] Thus, the subcellular localization and functional characteristics of OCTN1 as a cation transporter expressed in kidney are novel, and OCTN1 may be at least partially involved in the excretion of cationic xenobiotics as well as endogenous metabolites into urine.

Until now, apical membrane cation transporters have been reported to be proton/organic cation antiporters not affected by membrane potential, based on studies in rats, rabbits, and the cultured LLC-PK1 cells. There are only a few papers on organic cation transport in the human kidney. Ott et al. examined transport of organic cation such as NMN and TEA in human renal apical membrane vesicles and suggested that NMN uptake is pH-dependent with some membrane potential effect.[34] Accordingly, there may be species differences in the renal cation transporter, and/or multiple transporters may be involved in this process. Indeed, an organic cation transporter distinct from the proton antiporter that was affected by ATP was reported,[36] and the presence of multiple organic cation transporters was also reported.[27] Accordingly, OCTN1 may not be major, but should be functional as a pH-dependent transporter of organic cations across the apical membrane in human kidney. As noted in a very recent report on basolateral membrane transport of organic cations in human tissues, characterization of transporters in the kidney has not been fully performed in humans.[37] Therefore, it is essential to characterize the apical membrane transport in human kidney to identify the key transporters.

Interestingly, although there may be minor species differences, OCTN1 and OCTN2 are expressed in various tissues, while OCT1 and OCT2 are expressed in kidney, liver, and intestine. A previous study on the tissue distribution of TEA in rats demonstrated that TEA accumulated in several tissues, including heart, gut, lung, and skin as well as liver and kidney in rats.[38] Since TEA is hydrophilic and does not readily cross the cell membrane without the participation of a specific transporter, these tissues presumably contain a TEA transporter. In rats, OCTN1 is expressed in lung, heart, small intestine, skin, liver, and kidney,[32] i.e., the tissues in which a high level of TEA was observed. Although OCTN2 is also capable of transporting organic cations,[16] it is not expressed in lung or heart in rats.[39] Furthermore, in these tissues, the proton gradient across the cell membrane may not be enough to drive organic cation transport via a proton/organic cation antiport mechanism. Therefore, organic cation/organic cation antiport via OCTN1 in lung, heart, skin, gut, and other tissues might be important pharmacologically for tissue distribution of cationic drugs. However, OCT3, which is expressed in a variety of tissues, may also contribute to the tissue distribution of cationic compounds, and further studies are needed to clarify the pharmacological roles of these organic cation transporters in tissues other than kidney or liver.[6]

From a physiological point of view, since carnitine transport activity of OCTN1 is much lower than that of OCTN2, it is expected that OCTN1 has endogenous substrates other than carnitine or cationic drugs. It was also demonstrated that simultaneous disruption of OCTN1 and OCTN2 genes in mice caused significant problems with lipid metabolism which were not observed after disruption of the OCTN2 gene alone,[40] suggesting that OCTN1 has important biological roles. Accordingly, further studies on the endogenous substrates for OCTN1 will be essential in clarifying the transport mechanism, as well as the physiological function, of OCTN1.

In summary, this study has clarified for the first time that OCTN1 is localized at the apical membrane of the renal proximal tubular epithelial cells and its functionality is affected by pH, membrane potential, and cations on the trans side. OCTN2 has already been shown to be expressed at the renal apical membrane and to function for reabsorption of carnitine, as well as secretion of cationic compounds, so OCTN1 is the second organic cation transporter known to be expressed in the apical membrane. Furthermore, the significant countertransport and broad tissue expression profile indicate that OCTN1 should contribute to the tissue distribution of cationic drugs, suggesting pharmacological relevance of OCTN1 in addition to elimination into urine.

Abbreviations Used

FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NMN, *N*-methylnicotinamide; pH_{in}, intravesicular pH; pH_{out}, extravesicular pH; PMSF, phenylmethanesulfonyl fluoride; TEA, tetraethylammonium.

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(36) McKinney, T. D.; Hosford, M. A. ATP-stimulated Tetraethylammonium Transport by Rabbit Renal Brush Border Membrane Vesicles. *J. Biol. Chem.* **1993**, *268*, 6886−6895.

(37) Pietig, G.; Mehrens, T.; Hirsch, J. R.; Cetinkaya, I.; Piechota, H.; Schlatter, E. Properties and Regulation of Organic Cation Transport in Freshly Isolated Human Proximal Tubules. *J. Biol. Chem.* **2001**, *276*, 33741−33746.

(38) Mintun, M.; Himmelstein, K. J.; Schroder, R. L.; Gibaldi, M.; Shen, D. D. Tissue Distribution Kinetics of Tetraethylammonium ion in the Rat. *J. Pharmacokinet. Biopharm.* **1980**, *8*, 373−409.

(39) Sekine, T.; Kusuhara, H.; Utsunomiya-Tate, N.; Tsuda, M.; Sugiyama, Y.; Kanai, Y.; Endou, H. Molecular Cloning and Characterization of High-affinity Carnitine Transporter From Rat Intestine. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 586−591.

(40) Zhu, Y.; Jong, M. C.; Frazer, K. A.; Gong, E.; Krauss, R. M.; Cheng, J. F.; Boffelli, D.; Rubin, E. M. Genomic Interval Engineering of Mice Identifies a Novel Modulator of Triglyceride Production. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1137−1142.