Oppositely directed H⁺ gradient functions as a driving force of rat H⁺/organic cation antiporter MATE1

Masahiro Tsuda, Tomohiro Terada, Jun-ichi Asaka, Miki Ueba, Toshiya Katsuura, and Ken-ichi Inui

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan

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Oppositely directed H⁺ gradient functions as a driving force of rat H⁺/organic cation antiporter MATE1. Am J Physiol Renal Physiol 292: F593–F598, 2007. First published October 17, 2006; doi:10.1152/ajprenal.00312.2006.—Recently, we have isolated the rat (r) H⁺/organic cation antiporter multidiug and toxin extrusion 1 (MATE1) and reported its tissue distribution and transport characteristics. Functional characterization suggested that an oppositely directed H⁺ gradient serves as a driving force for the transport of a prototypical organic cation, tetraethylammonium, by MATE1, but there is no direct evidence to prove this. In the present study, therefore, we elucidated the driving force of tetraethylammonium transport via rMATE1 using plasma membrane vesicles isolated from HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells). A 70-kDa rMATE1 protein was confirmed to exist in HEK-rMATE1 cells, and the transport of various organic cations including [14C]tetraethylammonium was stimulated in intracellular acidified HEK-rMATE1 cells but not mock cells. The transport of [14C]tetraethylammonium in membrane vesicles from HEK-rMATE1 cells exhibited the overshoot phenomenon only when there was an outwardly directed H⁺ gradient, as observed in rat renal brush-border membrane vesicles. The overshoot phenomenon was not observed in the vesicles from mock cells. The stimulated [14C]tetraethylammonium uptake by an H⁺ gradient [intravesicular H⁺ concentration ([H⁺]v) > extravesicular H⁺ concentration ([H⁺]o)] was significantly reduced in the presence of a protonophore, carbonyl cyanide m-trifluoromethoxyphenylhydrazone (FCCP). [14C]tetraethylammonium uptake was not changed in the presence of valinomycin-induced membrane potential. These findings definitively indicate that an oppositely directed H⁺ gradient serves as a driving force for tetraethylammonium transport via rMATE1, and this is the first demonstration to identify the driving force of the MATE family. The present experimental strategy is very useful in identifying the driving force of cloned transporters whose driving force has not been evaluated.

MATERIALS AND METHODS

Materials. [14C]Levofloxacin (1.07 GBq/mmol) was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). [14C]Tetraethylammonium bromide (2.035 GBq/mmol), [14C]creatinine (2.035 GBq/mmol), [14C]proacainamide (2.035 GBq/mmol), [3H]quinidine (740 GBq/mmol), [3H]quinine (740 GBq/mmol), L-[N-methyl-3H]nicotine (3.145 TBq/mmol), and N-[methyl-14C]nicotine (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, Missouri). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: K. Inui, Dept. of Pharmacy, Kyoto Univ. Hosp. Sakyoku, Kyoto 606-8507, Japan (e-mail: inui@kuhp.kyoto-u.ac.jp).

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MO), [1-14C]metformin (962 MBq/mmol), [1-14C]guanidine hydrochloride (1,961 GBq/mmol), [8-3H]acyclovir (110 GBq/mmol), and [8-3H]ganciclovir (370 GBq/mmol) were purchased from Moravec Biochemicals (Brea, CA). [9-1H]methyl-1-phenylpiridine acidate (2.7 ΤBq/mmol), [14H]estrone sulfate ammonium salt (2.1 TBq/mmol), and [1-14C]-p-aminophenipurate (1.9 GBq/mmol) were purchased PerkinElmer Life Analytical Sciences (Boton, MA). [N-ethyl-3H]cimetidine (451 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals were used of the highest purity available.

Cell culture and transfection. HEK293 cells (American Type Culture Collection CRL-1573) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO2-95% air at 37°C. pcDNA 3.1 (+) containing cDNA encoding rMATE1 or empty vector was transduced into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, the cells were split in complete medium containing G418 (0.5 mg/ml, Nacalai Tesque, Kyoto, Japan) at a dilution of 1:200. Fifteen days after transfection, single colonies were picked out. Cells expressing rMATE1 (HEK-rMATE1) cells were selected by measuring [14C]tetraethylammonium transport by rMATE1 or HEK-rMATE1 cells were used as controls. These transfecants were maintained in complete medium with G418 (0.5 mg/ml).

Uptake experiments by HEK-rMATE1 cells. The cellular uptake of [14C]tetraethylammonium was measured by using monolayers grown on polyn-l-lysine-coated 24-well plates as reported previously with some modifications (13, 20, 22). Briefly, the cells were preincubated with 0.2 ml of incubation medium, pH 7.4 (in mM: 145 NaCl, 3 KCl, 1 CaCl2, 0.5 MgCl2, 5 D-glucose, and 5 HEPES) containing 30 mM NH4Cl for 20 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium pH 7.4 containing each radiolabeled compound was added. After an appropriate period of incubation, the medium was aspirated, and the monolayers were gently washed twice with 1 ml of ice-cold incubation medium (pH 7.4). The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in alisquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

Preparation of membrane vesicles from HEK-rMATE1 cells. Plasma membrane vesicles were prepared according to previous reports (6, 9). HEK-rMATE1 or HEK-pcDNA cells were seeded on 100-mm plastic dishes (4 × 109 cells/dish), and 20 or 40 dishes were used to prepare membrane vesicles in a single preparation. All procedures were performed at 4°C. At the third day after seeding, HEK-rMATE1 or HEK-pcDNA cells were washed with PBS and scraped with a rubber policeman into PBS. The cell suspension was centrifuged at 200 g for 10 min, suspended in 20 ml of PBS, and recentrifuged at 200 g for 10 min. The packed cell pellet was resuspended in 20 vol of 250 mM mannitol/10 mM HEPES-Tris (pH 7.5)/0.5 mM MgCl2 (buffer A), and the cells were gently suspended with five strokes of a loose-fitting Dounce homogenizer. The washed cell suspension was placed in a nitrogen cavitation bomb (Parr Instrument) at 700 lb/in.2 for 15 min. After the homogenate was collected, K-EDTA (pH 7.5) was added to a final concentration of 1 mM. The homogenate was centrifuged at 750 g for 15 min, and the supernatant was centrifuged at 20,000 g for 15 min. The supernatant was centrifuged at 100,000 g for 60 min. The pellet was resuspended in 100 mM mannitol/10 mM MES-KOH (pH 6.0; experimental buffer) or 100 mM mannitol/10 mM HEPES-KOH (pH 7.5; experimental buffer) and centrifuged again at 100,000 g for 60 min. The pellet was suspended in the same experimental buffer (pH 6.0 or 7.5) by sucking the suspension 10 times through a fine needle (4–10 mg protein/ml). KCl (pH 6.0 or 7.5) was added to a final concentration of 100 mM.

Transport experiments by membrane vesicles. The uptake of [14C]tetraethylammonium by membrane vesicles was measured by a rapid filtration technique with a slight modification (8, 19). In the regular assays, the reaction was initiated rapidly by adding 80 μl of buffer, containing 31.25 μM [14C]tetraethylammonium, to 20 μl of membrane vesicle suspension at 25°C. After specified periods, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution containing (in mM) 150 KCl, 20 HEPES-Tris (pH 7.5), 0.1 MgCl2, and 1 tetraethylammonium. The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm in diameter), and the filters were washed with 5 ml of ice-cold stop solution. The radioactivity of [14C]tetraethylammonium trapped in membrane vesicles was determined using an ACS II (Amersham Biosciences) by liquid scintillation counting. The protein content was determined by the method of Bradford (1) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine γ-globulin as a standard.

Western blot analysis. Polyclonal antibody was raised against a synthetic peptide corresponding to the intracellular domain of rMATE1 (CQQAQVHANLKVN, no. 465–477) (13). Brush-border membrane vesicles from rat kidney cortex were prepared as described previously (12). Membrane fractions were separated by SDS-PAGE and analyzed by Western blotting as described previously (17, 21).

Data analysis. Data were analyzed statistically with a one-way analysis of variance followed by Scheffe's test and are expressed as means ± SE.

RESULTS

Generation of HEK-rMATE1 cells. First, we generated and characterized HEK293 cells stably expressing rMATE1. As shown in Fig. 1, an immunoreactive protein with a molecular weight of ~70 kDa was detected in HEK-rMATE1 cells and rat renal brush-border membranes but not in HEK-pcDNA cells. The functional expression of rMATE1 was assessed by measuring the.

Fig. 1. Western blot analysis of rat renal brush-border membranes and plasma membranes obtained from HEK-rat multidrug and toxin extrusion 1 (rMATE1) and HEK-pcDNA cells. Renal brush-border membranes (20 μg) and plasma membranes (5 or 20 μg) obtained from HEK-rMATE1 and HEK-pcDNA cells were separated by SDS-PAGE (10%) and blotted onto polyvinylidene difluoride membranes. The antiserum for rMATE1 (1:1,000) was used as a primary antibody. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and the strips of blots were visualized by chemiluminescence on X-ray film. The arrowhead indicates the position of rMATE1. Lanes were as follows: lane 1, rat renal brush-border membranes; lane 2, HEK-rMATE1 (5 μg); lane 3, HEK-rMATE1 (20 μg); lane 4, HEK-pcDNA (5 μg); and lane 5, HEK-pcDNA (20 μg).
uptake of \([^{14}\text{C}]\)tetraethylammonium in the HEK-rMATE1 cells under the intracellular acidified conditions caused by NH\(_4\)Cl pretreatment. A time- and concentration-dependent uptake of \([^{14}\text{C}]\)tetraethylammonium by HEK-rMATE1 cells was observed (Fig. 2, A and B). \([^{14}\text{C}]\)tetraethylammonium uptake by HEK-rMATE1 cells exhibited saturable kinetics, and an apparent \(K_m\) value of 304 ± 80 \(\mu\)M was calculated from three separate experiments. When the extracellular pH was changed from 6.0 to 8.5, a bell-shaped pH profile of \([^{14}\text{C}]\)tetraethylammonium uptake via rMATE1 was observed, and the uptake was greatest at pH 7.5 and lowest at pH 6.0 (Fig. 2C).

Uptake of various compounds by HEK-rMATE1 cells. We then examined the substrate specificity of rMATE1. As shown in Fig. 3, rMATE1 mediated the transport of various organic cations with different chemical structures such as \([^{14}\text{C}]\)tetraethylammonium, \([^3\text{H}]\)1-methyl-4-phenylpyridinium acetate, \([^3\text{H}]\)cimetidine, and \([^{14}\text{C}]\)metformin. The transport of other organic cations such as \([^{14}\text{C}]\)procainamide, \([^{14}\text{C}]\)creatinine, and \([^{13}\text{C}]\)guanidine was greater in HEK-rMATE1 cells than in HEK-pcDNA cells, although the stimulation was not remarkable.

Characteristics of \([^{14}\text{C}]\)tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. Next, we performed transport experiments using plasma membrane vesicles isolated from HEK-rMATE1 cells and HEK-pcDNA cells. In the presence of an H\(^+\) gradient [intravesicular H\(^+\) concentration ([H\(^+\)]\(_\text{in}\)) > extravesicular H\(^+\) concentration ([H\(^+\)]\(_\text{out}\))], a marked
stimulation of $[\mathrm{^{14}C}]$tetraethylammonium uptake (overshoot phenomenon) was observed in membrane vesicles from HEK-rMATE1 cells, but not in those from HEK-pcDNA cells (Fig. 4). The overshoot phenomenon disappeared in the presence of an excess of cold tetraethylammonium.

Driving force for $[\mathrm{^{14}C}]$tetraethylammonium transport by membrane vesicles from HEK-rMATE1 cells. To elucidate the driving force of tetraethylammonium transport by rMATE1, we performed $[\mathrm{^{14}C}]$tetraethylammonium transport experiments using membrane vesicles from HEK-rMATE1 cells. As shown in Fig. 5, the presence of an $\mathrm{H}^+$ gradient ($[\mathrm{H}^+]_{\text{in}} > [\mathrm{H}^+]_{\text{out}}$) induced a marked stimulation of $[\mathrm{^{14}C}]$tetraethylammonium uptake against the concentration gradient. On the other hand, no stimulation of $[\mathrm{^{14}C}]$tetraethylammonium uptake was observed in the absence of the gradient or in the presence of the reverse gradient ($[\mathrm{H}^+]_{\text{in}} < [\mathrm{H}^+]_{\text{out}}$). The final amount of $[\mathrm{^{14}C}]$tetraethylammonium taken up in the presence of the $\mathrm{H}^+$ gradient ($[\mathrm{H}^+]_{\text{in}} > [\mathrm{H}^+]_{\text{out}}$) was not so different from that attained in the absence of the gradient or in the presence of the reverse gradient ($[\mathrm{H}^+]_{\text{in}} < [\mathrm{H}^+]_{\text{out}}$).

To further evaluate the effect of an outwardly directed $\mathrm{H}^+$ gradient on $[\mathrm{^{14}C}]$tetraethylammonium uptake, the influence of a protonophore, FCCP, was examined. As shown in Fig. 6A, the initial rate of $[\mathrm{^{14}C}]$tetraethylammonium uptake in the presence of an $\mathrm{H}^+$ gradient ($[\mathrm{H}^+]_{\text{in}} > [\mathrm{H}^+]_{\text{out}}$) was markedly...
reduced by FCCP, although the values at 30 min were similar in the absence or presence of FCCP.

To determine whether $^{14}$C-tetraethylammonium uptake depends on membrane potential, the effect of a K$^+$ diffusion potential generated by valinomycin on $^{14}$C-tetraethylammonium uptake was examined. As shown in Fig. 6B, the H$^+$ gradient-stimulated $^{14}$C-tetraethylammonium uptake was not altered by the presence of valinomycin. Furthermore, we also examined the effect of a K$^+$ diffusion potential generated by valinomycin on rMATE1-mediated $^{14}$C-tetraethylammonium uptake in the absence of H$^+$ gradient (H$^+$)$_{in} = [H^+]_{out}$, pH 7.5). $^{14}$C-tetraethylammonium uptake was not significantly changed with or without valinomycin at 30 s (with valinomycin, 1.77 ± 0.21; without valinomycin, 1.84 ± 0.14 pmol/mg protein$^{-1}$·30 s$^{-1}$; n = 3) and at 1 min (with valinomycin, 3.10 ± 0.18; without valinomycin, 3.48 ± 0.68 pmol/mg protein$^{-1}$·min$^{-1}$; n = 3). These results indicate that the inside-negative membrane potential does not affect $^{14}$C-tetraethylammonium uptake by rMATE1, suggesting the electroneutral antiport of H$^+$ and $^{14}$C-tetraethylammonium.

**DISCUSSION**

Transport studies in brush-border and basolateral membrane vesicles from renal epithelial cells have been successfully utilized to characterize a number of transport systems under well-defined in vitro conditions. The membrane vesicle studies are particularly useful for identifying the driving forces of secondary active transport systems, compared with other analyses. This is because the ionic composition inside or outside membrane vesicles is easily manipulated, and ion gradients and membrane potential can be provided artificially. In fact, it was clearly demonstrated that organic cation transport systems at the renal brush-border membranes are driven by an outwardly directed H$^+$ gradient (4, 19, 23). Recent cloning and functional studies of MATE1 from various species have suggested that an oppositely directed H$^+$ gradient was a driving force of tetraethylammonium transport by MATE1 (3, 13, 15, 20), but there had been no evidence of a direct coupling of organic cation transport to H$^+$.

In the present study, by using membrane vesicles from HEK-rMATE1 cells, we provide the first direct evidence that MATE1 mediates the H$^+$-coupled uphill transport of $^{14}$C-tetraethylammonium. Furthermore, this stimulation disappeared in the presence of a protonophore, FCCP, indicating that MATE1 functions as the H$^+$/organic cation antiporter. The K$^+$ diffusion potential generated by valinomycin had no effect on $^{14}$C-tetraethylammonium uptake by membrane vesicles from HEK-rMATE1 cells with or without an H$^+$ gradient. This is consistent with a report that the tetraethylammonium uptake by brush-border membrane vesicles was not enhanced by inside-negative membrane potential (19). Taken together, it is suggested that the antiport of H$^+$ and tetraethylammonium via rMATE1 is electroneutral and that the stoichiometry might be 1:1.

In our previous study (20), using rMATE1, transiently expressing cells without NH$_4$Cl pretreatment, we assessed the time course of $^{14}$C-tetraethylammonium uptake (pH 8.4), pH profile of $^{14}$C-tetraethylammonium uptake, and substrate specificity at the pH 8.4. In the present study, using HEK-rMATE1 cells with NH$_4$Cl pretreatment, the transport characteristics for rMATE1 were analyzed by $^{14}$C-tetraethylammonium (pH 7.4) or various compounds (pH 7.4). It was reported that the intracellular pH of HEK293 cells is ~7.2 and transiently acidified to 6.0–6.5 by NH$_4$Cl pretreatment (11). These distinct experimental conditions may have affected the different transport characteristics of rMATE1. For example, we previously reported that the intracellular accumulation of $^{14}$C-tetraethylammonium via rMATE1 showed a time-dependent increase. In the present study, $^{14}$C-tetraethylammonium intracellular accumulation by rMATE1 peaked at 30–60 s and then gradually decreased. This may be due to the consumption of the outward H$^+$ gradient within 30–60 s and subsequent back flux of $^{14}$C-tetraethylammonium via MATE1. In addition, $[^3]$H1-methyl-4-phenylpyridinium acetate and $[^14]$C-procainamide were transported by rMATE1 in the present study, but not in the previous study. This may be due to the lack of a strong enough driving force to transport $[^3]$H1-methyl-4-phenylpyridinium acetate and $[^14]$C-procainamide in the previous conditions.

In conclusion, we generated HEK293 cells stably expressing rMATE1, and clearly demonstrated that the driving force of tetraethylammonium transport by rMATE1 is an oppositely directed H$^+$ gradient using membrane vesicles from this stable transfectant. These findings can provide important information about the renal tubular secretion of organic cations, and these experimental strategies may be useful for elucidating the mechanisms of action used by single transporters in heterologous expression systems.

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