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

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Submitted 9 August 2006; accepted in final form 14 October 2006

Recently, Moriyama and co-workers (3, 15) have identified human (h) and mouse MATE1 and MATE2, which are orthologs of the multidrug and toxin extrusion (MATE) family of bacteria. They demonstrated that MATE1 was predominantly expressed at the luminal membranes of the urinary tubules and bile canaliculi and transported tetraethylammonium, a prototypical organic cation, in a pH-dependent manner (3, 15). We also isolated cDNAs for rat (r) MATE1 (20) and the human kidney-specific isoform MATE2-K (13). rMATE1 was significantly expressed in the kidney and placenta, but not in the liver, and real-time PCR analyses of microdissected nephron segments showed that rMATE1 was expressed in the proximal convoluted and straight tubules (20). On the other hand, hMATE2-K was only expressed in the kidney and was located at the brush-border membranes of renal proximal tubular cells (13). By conducting functional analyses, we showed that rMATE1 and hMATE2-K can transport a wide variety of organic cations including tetraethylammonium, \(N\)-methyl-N-nicotinamide, and metformin (13, 20). These characteristics of MATE1 are similar to those of the H\(^+\)/organic cation antiport system revealed by renal brush-border membrane vesicle studies (4, 14, 18, 19, 23).

MATE1 exhibited pH-dependent transport of tetraethylammonium in cellular uptake and efflux studies, and intracellular acidification by NH\(_4\)Cl pretreatment stimulated tetraethylammonium transport (3, 13, 15, 20), suggesting that MATE1 utilized an oppositely directed H\(^+\) gradient as a driving force. However, these analyses are not enough to prove the H\(^+\)/tetraethylammonium antiport mechanism of MATE1, because it is possible that the pH-dependent transport of tetraethylammonium by MATE1 is regulated not by an H\(^+\) gradient but by pH itself. Accordingly, in addition to the data obtained using the cell culture model, we need more direct evidence that an H\(^+\) gradient is the driving force for MATE1.

In the present study, we developed HEK293 cells stably expressing rMATE1 (HEK-rMATE1 cells) and elucidated the driving force of rMATE1 by uptake studies using plasma membrane vesicles from HEK-rMATE1 cells for the first time.

**MATERIALS AND METHODS**

**Materials.** \[^{14}C\]levofloxacin (1.07 GBq/mmol) was kindly provided by Daiichi Pharmaceutical (Tokyo, Japan). \[^{14}C\]tetraethylammonium bromide (2.035 GBq/mmol), \[^{14}C\]creatinine (2.035 GBq/mmol), \[^{14}C\]procainamide (2.035 GBq/mmol), \[^{3}H\]quinidine (740 GBq/mmol), \[^{3}H\]quinine (740 GBq/mmol), 1-\(^{[N\text{-methyl-}^{3}H]\text{]}}\text{carnitine (3.145 TBq/mmol), and [N\text{-methyl-}^{14}C\text{]nicotine (2.035 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, Missouri).}

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MO), [14C]metformin (962 MBq/mmole), [14C]guanidine hydrochloride (1,961 GBq/mmole), [8-14H]acetylimidazole (110 GBq/mmole), and [8-14H]acetaldehyde (370 GBq/mmole) were purchased from Moravec Biochemicals (Brea, CA). [H]-methyl-4-phenylpyridinium acetate (2.7 TBq/mmole), [H]-estrone sulfate ammonium salt (2.1 TBq/mmole), and [14C]-l-aminophosph氛ate (1.9 GBq/mmole) were purchased from PerkinElmer Life Analytical Sciences (Boton, MA). [N-methyl-[H]-cimetidine (451 GBq/mmole) was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals were 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 transfection into HEK293 cells using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h after transfection, the cells were split into 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 uptake. Cells transfected with empty vector (HEK-pcDNA 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 polycarbonate-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, 2 CaCl2, 0.5 MgCl2, 5 D-glucose, and 5 HEPES) containing 30 mM mannitol/10 mM HEPES-KOH (pH 7.5; experimental buffer) were centrifuged at 100,000 g for 10 minutes and HEK-pcDNA cells. Renal brush-border membranes but not in HEK-pcDNA cells. The functional expression of rMATE1 was assessed by measuring the 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 HgCl2, 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 Scheffé’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 antiserum for rMATE1 (CQQAQVHANLKVN, no. 465–477) (13). The following membranes (5 or 20 mg) of HEK-rMATE1 (5 mg) and plasma 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 antigen 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 \([^{14}C]\)tetraethylammonium in the HEK-rMATE1 cells under the intracellular acidified conditions caused by NH\(_4\)Cl pretreatment. A time- and concentration-dependent uptake of \([^{14}C]\)tetraethylammonium by HEK-rMATE1 cells was observed (Fig. 2, A and B). \([^{14}C]\)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 \([^{14}C]\)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 \([^{14}C]\)tetraethylammonium, \([^{3}H]\)1-methyl-4-phenylpyridinium acetate, \([^{3}H]\)cimetidine, and \([^{14}C]\)metformin. The transport of other organic cations such as \([^{14}C]\)procainamide, \([^{14}C]\)creatinine, and \([^{13}C]\)guanidine was greater in HEK-rMATE1 cells than in HEK-pcDNA cells, although the stimulation was not remarkable.

**Characteristics of \([^{14}C]\)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\(^+\)]\(_{in}\)) > extravesicular H\(^+\) concentration ([H\(^+\)]\(_{out}\))), a marked...
stimulation of [14C]tetraethylammonium uptake (overshoot phenomenon) was observed in membrane vesicles from HEK-rMATE1 cells, but not in those from HEK-pcDNA cells (Fig. 4).

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

To further evaluate the effect of an outwardly directed H+ gradient on [14C]tetraethylammonium uptake, the influence of a protonophore, FCCP, was examined. As shown in Fig. 6A, the initial rate of [14C]tetraethylammonium uptake in the presence of an H+ gradient ([H+]\text{in} > [H+]\text{out}) was markedly lower than in the absence of the gradient or in the presence of the reverse gradient ([H+]\text{in} < [H+]\text{out}).

Fig. 4. Time course of [14C]TEA uptake by membrane vesicles from HEK-pcDNA and HEK-rMATE1 cells. The uptake of [14C]TEA by membrane vesicles from HEK-pcDNA cells (○, △) and HEK-rMATE1 cells (●, ▲) was examined in the absence (○, ●) or presence (△, ▲) of 10 mM TEA. Membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [14C]TEA was examined in the experimental buffer containing 31.25 μM [14C]TEA and 100 mM KCl at pH 7.5 in the absence or presence of 10 mM TEA. Each point represents the mean ± SE of 3 determinations.

Fig. 5. Effect of H+ gradient on [14C]TEA uptake by membrane vesicles from HEK-rMATE1 cells. Membrane vesicles were prepared in the experimental buffer at pH 6.0 or pH 7.5. The uptake of [14C]TEA was examined in the experimental buffer containing 31.25 μM [14C]TEA and 100 mM KCl at pH 7.5 in the absence (○, △) or presence (●, ▲) of 10 mM TEA. Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments. pH\text{in}, intravesicular pH; pH\text{out}, extravesicular pH.

Fig. 6. Effect of FCCP (A) and valinomycin (B) on [14C]TEA uptake in the presence of an outwardly directed H+ gradient by membrane vesicles from HEK-rMATE1 cells. A: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [14C]TEA was examined in the experimental buffer containing 31.25 μM [14C]TEA and 100 mM KCl at pH 6.0 in the absence (○, △) or presence (●, ▲) of 10 mM FCCP. Each point represents the mean ± SE of 3 determinations. The figure is a representative of 2 separate experiments. B: membrane vesicles were prepared in the experimental buffer at pH 6.0. The uptake of [14C]TEA was examined in the experimental buffer containing 31.25 μM [14C]TEA and 100 mM CsCl at pH 7.5 in the absence (○, △) or presence (●, ▲) of 8 μM valinomycin. Each point represents the mean ± SE of 3 determinations. The figure is representative of 2 separate experiments.
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$^+$)$_{\text{in}}$ = [H$^+$]$_{\text{out}}$, 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}H]$1-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}H]$1-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.

**GRANTS**

This work was supported by the 21st Century COE Program “Knowledge Information Infrastructure for Genome Science,” a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan. J. Asaka is supported as a research assistant by the 21st Century COE program “Knowledge Information Infrastructure for Genome Science.”

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