The multidrug transporter MATE1 sequesters OCs within an intracellular compartment that has no influence on OC secretion in renal proximal tubules

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The multidrug transporter MATE1 sequesters OCs within an intracellular compartment that has no influence on OC secretion in renal proximal tubules. Am J Physiol Renal Physiol 310: F57–F67, 2016. First published November 4, 2015; doi:10.1152/ajprenal.00318.2015.—Secretion of organic cations (OCs) across renal proximal tubules (RPTs) involves basolateral OC transporter (OCT)2-mediated uptake from the blood followed by apical multidrug and toxin exchanger (MATE)1/2-mediated efflux into the tubule filtrate. Whereas OCT2 supports electrogenic OC uniport, MATE is an OC/H+ exchanger. As assessed by epifluorescence microscopy, cultured Chinese hamster ovary (CHO) cells that stably expressed human MATE1 accumulated the fluorescent OC N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium (NBD-MMA) in the cytoplasm and in a smaller, punctate compartment; accumulation in human OCT2-expressing cells was largely restricted to the cytoplasm. A second intracellular compartment was also evident in the multicompartamental kinetics of efflux of the prototypic OC 1-methyl-4-phenylpyridinium (MPP) (10). We found the interaction of MATE1-mediated efflux in an examination of the influence of intracellular H+ concentration on the rate of efflux of the prototypic OC 1-methyl-4-phenylpyridinium (MPP) (10). We found the interaction of H+ with MATE1 to be symmetrical, with IC50 values of 12–15 nM, i.e., pH 7.8, for interaction with both the intracellular and extracellular faces of the transporter. This was not unexpected because protons titrate single carboxyl or imidazole residues and the three-dimensional structure of binding surfaces may have comparatively little influence on H+ binding kinetics. However, there is no reason to expect kinetic interactions of structurally complex organic molecules with transporters to be the same for influx and efflux, i.e., to be symmetrical (34). Indeed, rat Oct2 (Slc22a2) has been shown to have markedly asymmetrical interactions with (at least) some inhibitory ligands (37).

The typical emphasis on OC influx in studies of MATE1 activity is certainly influenced by the fact that the measurement of efflux from cells is challenging; cells are small, and the rate can change rapidly. The measurement of MATE1-mediated efflux is further complicated by what appears to be the presence of a second intracellular compartment of accumulated OCs in cultured cells that heterologously express MATE1 (10). In fact, evidence for intracellular sequestration of OC mediated by OC/H+ exchange is not new; Pritchard and colleagues (25) have shown that isolated endosomal membrane vesicles from rat renal tubules display H+ gradient-dependent tetraethylammonium (TEA) transport. They found that endosomes, with acidic interiors due to activity of V-type H+-ATPase, possess...
OC/H⁺ exchange with characteristics similar to those found in the luminal membrane of RPT cells. They further suggested that intracellular sequestration of OC within endosomes could play an important role in renal OC secretion by fusing with the apical plasma membrane and delivering their contents to the tubular filtrate.

We reasoned that, if characterizing MATE1-mediated OC efflux is an important goal, then understanding the nature of intracellular sequestration of OCs in cells that express this transporter will be required. Here, we confirm the presence of an intracellular compartment in MATE1-expressing cells, including intact RPT cells, that accumulates OCs by a process that is dependent on the activity of V-type H⁺-ATPase. Furthermore, we show that, whereas this process does significantly influence the accumulation of OCs in intact RPTs as well as in cultured cells that express MATE1, it does not influence the net rate of OC secretion by RPTs.

MATERIALS AND METHODS

Chemicals. [3H]MPP (specific activity: 80 Ci/mmol) and [3H]N,N,N-trimethyl-2-[methyl(7-nitrobenzoyl)[il]2,5-[oxadiazole-l-y1] amino]ethanaminium iodide [NBD-MTMA; specific activity: 85 Ci/ mmol (5)] were synthesized by the Department of Chemistry and Biochemistry of the University of Arizona. Unlabeled NBD-MTMA was synthesized by the Synthesis Core of the Southwest Environmental Health Sciences Center/Department of Chemistry of the University of Arizona (1). MPP, Ham’s F-12 Kaighn’s modified medium, and DMEM were obtained from Sigma-Aldrich. Other reagents were obtained from National Institutes of Health (NIH)/H9262, 1 Na lactate, 1 Na malate, and 0.9 glycine; pH 7.4 or 8.5) at room temperature. For time course experiments, cells were incubated in 200 μl of Waymouth’s buffer containing ~14 nM [3H]MPP or [3H]NBD-MTMA for 30 s to 15 min with 1, 5, or 10 μM BAF. To stop the transport process, each well was aspirated and rinsed three times with 1 ml of ice-cold Waymouth’s buffer. Cells were then solubilized in 200 μl of 0.5 N NaOH with 1% SDS and gently shaken for 30 min. For each sample, 100 μl of 1 N HCl was added to neutralize the cell lysate, and aliquots of 250 μl were placed in liquid scintillation vials later filled with 3 ml of scintillation cocktail (MP Biomedicals) and assessed for radioactivity using liquid scintillation spectrometry (LS6000IC, Beckman). Experiments that measured transport in cells grown in 96-well plates used an automatic fluid aspirator/dispenser (model 406, BioTek, Winooski, VT). Plates containing culture media were placed in the unit and automatically rinsed/aspirated three times with Waymouth’s buffer at room temperature, after which transport buffer (60 μl) was automatically introduced into each well. After the experimental incubation, the transport reaction was stopped by the rapid addition (and simultaneous aspiration) of ~750 μl of cold (4°C) Waymouth’s buffer. After final aspiration of the cold stop, 200 μl of scintillation cocktail (Microscint 20, Perkin-Elmer, Waltham, MA) were added to each well, and the plates were sealed (Topseal-A, Perkin-Elmer) and allowed to sit for at least 2 h before radioactivity was assessed in a 12-channel, multwell scintillation counter (Wallac Trilux 1450 Microbeta, Perkin-Elmer). In experiments measuring MATE1-mediated transport under the condition of an outwardly directed H⁺ gradient, cells were first preincubated for 20 min in 20 mM NH₄Cl. Transport was initiated by aspirating this medium and replacing it with NH₄Cl-free medium (thereby establishing the outwardly directed H⁺ gradient (17, 27)), containing the radiolabeled substrate, as described above.

Efflux experiments with cultured cells. CHO cells were plated in 35-mm cell culture dishes (Falcon) at 2 × 10⁶ cells/plate sufficient to reach confluence within 24 h (or within 48 h if plated at 1.2 × 10⁶ cells). Before the experiments, cells were rinsed twice with 300 μl of Waymouth’s buffer (pH 7.4) and then incubated for 20 min in Waymouth’s buffer (pH 8.5) containing 30 nM [3H]MPP and ~35 nM [14C]mannitol or [3H]NBD-MTMA and ~35 nM [14C]mannitol. To initiate efflux of the labeled substrate, the “loading buffer” was aspirated, and 500 μl of Waymouth’s buffer (pH 7.4) was added (time 0). Efflux was monitored by collecting this volume and immediately replacing it with an equal volume of Waymouth’s buffer every 10 s. Scintillation cocktail was added to the “effluent” sample, and radioactivity was determined. After 3 min, efflux was stopped, and the remaining intracellular labeled substrate was determined by lysing the cells in 600 μl of 0.5 N NaOH with 1% SDS. After gentle shaking for 30 min, 300 μl of 1.0 N HCl were used to neutralize the resulting lysate. Samples (900 μl) were placed in liquid scintillation vials and filled with 3 ml of scintillation cocktail; each sample was then transferred to a liquid scintillation counter to determine the amount of radioactivity.

Uptake experiments with isolated proximal tubules. Tubules were transferred to a multiwell, temperature-controlled Lucite chamber, and images were taken for future measurements of tubule length. For the measurement of peritubular (basolateral) uptake into nonperfused tubules, control and test uptake solutions were added to separate wells and covered with water-saturated light mineral oil to prevent evaporation. Uptake solutions consisted of [3H]MPP or [3H]NBD-MTMA in rabbit bicarbonate buffer (pH 7.4) with BAF (5 or 10 μM) added to the test well. The temperature was then brought to 37°C, and uptakes were performed as previously described (14). Briefly, individual tubules were picked up on a glass needle and transferred into
the control or test solution. After immersion for 5 min (MPP) or 10 min (NBD-MTMA), the tubule was transferred to a well in a Nunc plate containing 7 μl of 1 N NaOH (oil covered) for a 1-h extraction period. The 5-min uptake time was chosen for MPP based on preliminary time courses that showed uptake to be still relatively linear and provide counts sufficient for meaningful calculations and results (data not shown). NBD-MTMA required a longer time to accumulate sufficient counts. Uptakes were run alternating the different solutions to minimize the effect on transport of time since dissection. Extracts were then transferred to 0.2 ml water in a 5-ml scintillation vial, and 3 ml Ecolite (MP Biomedicals) was added. Accumulated radioactivity was determined by liquid scintillation spectrometry (LS6000, Beckman-Coulter).

Secretion by isolated proximal tubules. Tubules were transferred to a temperature-controlled Lucite chamber and covered with water-saturated light paraffin oil. The bathing solution was changed to rabbit bicarbonate buffer with 3% dextran, and the oil surface was continuously gassed with 95% O2-5% CO2. Perusptions were carried out as previously described (12). Briefly, rabbit bicarbonate buffer was introduced into the perfusion pipette and allowed to run several minutes before the tubule was connected for perfusion. The tubule (length of ~1.2 mm) was hooked up to the perfusion and collection pipettes. When steady accumulation of the perfusate in the collection pipette was observed, the temperature was brought to 37°C, and the first control solution was exchanged into the bath. The perfusate with its accumulated substrate was collected every 5 min with a precalibrated (in nl/mm) sampling pipette and placed in a scintillation vial containing 0.2 ml water. The rate of perfusion, as established by a pressure head of ~50 mmHg, was typically between 25 and 30 nl/min. At the end of the control period, the bath was replaced with a solution containing the test agent (5 μM BAF or 1 mM TEA), the collection pipette was emptied, and 5-min sample collections were resumed. The final control collections were made in the same manner. The tubule length in the bathing solution was measured, 3 ml Ecolite (MP Biomedicals) was added to the sample vials, and accumulated radioactivity was determined by liquid scintillation spectrometry.

Fluorescence microscopy. hMATE1- or hOCT2-expressing CHO cells or isolated single rabbit RPTs were preloaded with 20 μM NBD-MTMA with and without 10 μM BAF at 37°C for 20 min. Cells were then rinsed and transferred to ice-cold Waymouth’s buffer at pH 7.4. Cells/tubules were transferred to a fluorescence microscope equipped with a 41001HQ filter (excitation: 400 nm and emission: 650 nm, Chroma Technology), at which point images were collected.

Immunocytochemistry. CHO cells that stably expressed a COOH-terminal V5 epitope-tagged transporter (hMATE1 or hOCT2) were seeded onto coverslips. Immunocytochemistry was generally performed on a confluent monolayer 24 h after cells were plated. Cells were fixed in ice-cold 100% methanol for 20 min, washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7; all washes were done in triplicate), and then incubated for 1 h with anti-V5 antibody (Invitrogen) diluted 1:500 in PBS. Cells were then incubated for 1 h in the dark with FITC-conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:1,000 in PBS. To visualize the nuclei, cells were treated with propidium iodide (5 μg/ml) for 10 min. Cells were washed again, and the coverslips were mounted onto microscope slides using Dako fluorescent mounting media (Dako, Carpinteria, CA). An confocal microscope (Nikon PCM 2000 scan head fitted to a Nikon E800 microscope) was used for the detection of immunoreactinity in CHO cells. Separate experiments showed that wild-type CHO cells did not interact with the V5 antibody and that immunoreactivity was indicative of the expression of hMATE1 or hOCT2 protein (24, 43).

Data analysis. Results are presented as means ± SE. Statistical analyses were performed using a two-tailed unpaired Student’s t-test. In some cases, data sets were compared using one- or two-way ANOVA (with Bonferroni post tests). Efflux data were described by one-phase decay (with plateau) or two-phase decay (with no plateau) analysis (Prism 5.03, GraphPad Software, San Diego, CA), and kinetic parameters of efflux were compared using an extra sum-of-squares F-test. In all cases, observed differences were considered significant when P < 0.05.

RESULTS

Time course of MATE1-mediated OC uptake and efflux. As an OC/H+ exchanger, the time course of MATE1-mediated MPP uptake is markedly influenced by extracellular and intracellular pH. Under normal “steady-state” conditions for CHO cells at room temperature, there is, effectively, no transmembrane pH gradient (extracellular pH of 7.4 vs. intracellular pH of ~7.5) (10). Figure 1 shows representative profiles of time-dependent uptake determined in the absence and presence of imposed pH gradients. Figure 1A shows that, in the absence of a pH gradient (transport buffer pH 7.4), MATE1-mediated rates of transport were comparatively low (clearance of ~1 μl/cm2 at 10 min). In contrast, Fig. 1B shows that the reduction of H+ concentration in the transport buffer (pH 8.5) reduced the inhibitory (competitive) effect that extracellular H+ exerts on uptake of the labeled substrate (39) while also providing an outwardly directed H+ gradient (36); both effects combined synergistically to cause about a fivefold increase in the MATE1-mediated accumulation of MPP. The acidification of intracellular pH by the ammonia pulse technique (see METHODS) caused a larger increase in the initial rate of MATE1-mediated transport (achieving a clearance of 6.7 μl·cm−2·min−1 within 10 s), suggesting that the outwardly directed H+ gradient under these conditions is kinetically more effective than the gradient produced by exposing cells to an external pH of 8.5. However, this effect was transient, with maximum uptake achieved within 60 s followed by a fall in accumulated substrate, suggesting a collapse of the imposed pH gradient (Fig. 1C). As a comparison, Fig. 1D shows the uptake for hOCT2, which is independent of pH (4). The remaining experiments of substrate uptake were performed at pH 7.4, ostensibly reflecting normal physiological conditions (i.e., without an ammonia pulse-imposed pH gradient). Figure 2 shows the kinetics of MATE1-mediated MPP transport at pH 7.4. Transport was adequately described by Michaelis-Menten kinetics. As determined in three separate experiments, the maximal flux for MPP transport (under non-pH gradient conditions) was 3.9 ± 1.5 pmol·cm−2·min−1, with a Michaelis constant (Km) of 45.1 ± 16.3 μM.

We showed previously that rates of MATE1-mediated efflux are also influenced by extracellular and intracellular pH, with efflux stimulated by either a decrease in extracellular pH or by increases in intracellular pH (10). Here, we examined in more detail the kinetic characteristics of MATE1-mediated efflux under normal steady-state pH conditions (approximately equal pH inside and out) and compared that profile with the profile observed for pH-insensitive OCT2. Figure 3 shows a comparison of the time course of MPP efflux from MATE1- or OCT2-expressing CHO cells that were preloaded for 20 min with [3H]MPP. In both cases, the loss of MPP from cells was dominated by a rapidly exchangeable compartment and a second compartment that lost substrate sufficiently slowly (~8–12 times slower than the fast compartment) that it could be modeled as a “plateau” reflecting effective sequestration within the cell (Fig. 3A) (7). When total cell MPP content was
corrected for this second, very slow compartment, efflux from both the MATE1- and OCT2-expressing cells was adequately described as first order (Fig. 3B), as expected for efflux from a cytoplasmic concentration that was likely to be much lower than the $K_t$ for mediated MPP efflux (we expand on this issue below).

Of particular relevance to the focus of the present study was the marked difference in the value of the plateau for MATE1- versus OCT2-expressing cells, i.e., the slowly exchanging compartments in cells that expressed the two different transporters; the retained pool of MPP in MATE1-expressing cells was almost three times larger than that observed for OCT2-expressing cells (36.2% of time 0 cell content, compared with 13.1% for MATE1- and OCT2-expressing cells, respectively ($P < 0.05$).

As noted above, we previously reported that a fraction of OC accumulated by MATE1-expressing CHO cells appears to be sequestered within a “punctate” intracellular compartment, as shown by the distribution of the fluorescent OC NBD-MTMA (10). Figure 4 confirmed that previous observation and shows a comparison of the distribution of NBD-MTMA in CHO cells that expressed either MATE1 or OCT2. After a 20-min exposure to 20 $\mu$M NBD-MTMA (Waymouth’s buffer, 37°C, pH 8.5), MATE1-expressing cells revealed a diffuse fluorescent signal in the cytoplasm and a much brighter signal within a population of small intracellular “vesicles” (Fig. 4, A and B). Immunocytochemistry of the MATE1-expressing cells showed that, in addition to substantial expression of transport protein in the plasma membrane, immunoreactive MATE1 protein was also distributed throughout the cytoplasm (Fig. 5A), consistent with the presence of functional MATE1 protein within an intracellular membranous compartment.

The spatial distribution of accumulated NBD-MTMA in MATE1-expressing cells (Fig. 4B) contrasted sharply with that observed in OCT2-expressing cells (20 min in 20 $\mu$M NBD-MTMA, Waymouth’s buffer, 37°C, pH 7.4), in which the signal was largely restricted to a diffuse, and substantially brighter, cytoplasmic compartment (Fig. 4C). Although a few small, bright “inclusions” were noted in some OCT2-expressing cells, the signal appeared similar to that in the larger, cytoplasmic compartment (Fig. 4C). As with MATE1, hOCT2-immunoreactive protein was primarily expressed in the plasma membrane, but was also evident throughout the cytoplasm (Fig. 5B). Thus, the lack of bright inclusions was not correlated with a lack of cytoplasmic OCT2 protein.

Previous work of Pritchard et al. (25) showed that endosomes isolated from the rat renal cortex contain OC/H$^+$ exchange activity that is stimulated by the addition of ATP. This result, when combined with additional evidence, led them to conclude that activation of endosomal V-type H$^+$-ATPase develops a proton gradient that, in turn, can stimulate OC/H$^+$ exchange activity.
exchange. To test the hypothesis that the accumulation of NBD-MTMA within an intracellular compartment in MATE1-expressing cells reflected the presence of MATE1 in the endosomal-lysosomal pathway (ELP) membrane and the influence of the outwardly directed H+/H11001 gradient, we determined the effect of BAF, an inhibitor of V-type H+/H11001-ATPase (13), on the spatial distribution of NBD-MTMA in CHO cells that expressed either MATE1 or OCT2. If MATE1 is functionally expressed in the ELP, then inhibition of V-type H+/H11001-ATPase should decrease the ability of MATE1 to accumulate NBD-MTMA to levels substantially greater than that in the cytoplasm; in contrast, there should be little or no effect on the distribution of NBD-MTMA in cells that express OCT2. As shown in Fig. 4, coexposure to 20 μM NBD-MTMA plus 10 μM BAF resulted in a marked decrease in the apparent number and intensity of punctate inclusions in MATE1-expressing cells (Fig. 4, E and F). We have previously shown that the fluorescence of NBD-MTMA is not influenced by changes in

Fig. 3. Time course of MPP efflux from hMATE1- and hOCT2-expressing CHO cells. A: after a 20-min preload of ~30 nM [3H]MPP, the amount of labeled MPP remaining in the cells was measured as a function of time after efflux was initiated in MPP-free buffer of pH 7.4. Each point is the mean [3H]MPP content (±SE) determined in four individual experiments, expressed relative to total cell MPP content at time 0. The curvilinearity of the semilog plot revealed that there were (at least) two compartments containing MPP involved in [3H]MPP efflux from hMATE1- and hOCT2-expressing CHO cells: a fast compartment and a slow compartment, here modeled as a “plateau.” The solid line represents the one-phase exponential decline in cell [3H]MPP (rate constant of efflux shown); the dashed line represents the plateau. B: single-phase (first order) decline in cell [3H]MPP content after correction for the plateau (see text for discussion). All samples were corrected for extracellular “carryover” using [14C]mannitol.

Fig. 4. A: uptake (20-min accumulation, WB, pH 8.5) of the fluorescent OC,N,N,N-trimethyl-2-[methyl[7-nitrobenzo[c][1,2,5]oxadiazol-4-yl]amino]ethanaminium (NBD-MTMA; 20 μM) in CHO cells expressing hMATE1. B: three-dimensional (3-D) fluorescence distribution analysis of 20 μM NBD-MTMA in hMATE1-expressing cells (x- and y-axes represent the region analyzed, as indicated by the square within the field of Fig. 3A; the z-axis represents fluorescence intensity). C: uptake (20-min accumulation, WB, pH 7.4) of NBD-MTMA (20 μM) in CHO cells that expressed hOCT2. D: 3-D fluorescence distribution analysis of 20 μM NBD-MTMA in hOCT2-expressing cells. E: uptake (20-min accumulation, WB, pH 8.5) of NBD-MTMA (20 μM) and bafilomycin (BAF; 20 μM) in CHO cells that expressed hMATE1. F: 3-D fluorescence distribution analysis of 20 μM NBD-MTMA and BAF (20 μM) in hMATE1-expressing cells. G: uptake (20-min accumulation, WB, pH 7.4) of NBD-MTMA (20 μM) and BAF (20 μM) in CHO cells that expressed hOCT2. H: 3-D fluorescence distribution analysis of 20 μM NBD-MTMA and BAF (20 μM) in hOCT2-expressing cells. 3-D fluorescence distribution analysis used ImageJ (National Institutes of Health; http://imagej.nih.gov/ij/, 1997–2014).
pH over the range of 5.5–8.5 (1), so the decrease in punctate fluorescence in response to BAF treatment presumably reflected a decrease in vesicle NBD-MTMA content. In contrast, OCT2-expressing cells displayed comparatively little change in the distribution of NBD-MTMA (Fig. 4, G and H) after BAF treatment.

The difficulty inherent in quantifying the differential effect of BAF on the accumulation of fluorescent OCs in MATE1-expressing cells versus OCT2-expressing cells led us to apply an independent test of the hypothesis that the significant fraction of OC accumulation in MATE1-expressing cells involves sequestration in an acidic compartment. As shown in Fig. 6A, 15-min accumulation of \( {^{13}}\text{H} \)NBD-MTMA at pH 8.5 was reduced 30% or 45% by coexposure to 1 or 5 \( \mu \)M BAF, respectively (\( P < 0.05 \)). Similarly, 15-min accumulation of \( {^{3}}\text{H} \)MPP was reduced 36% or 45% by 1 or 5 \( \mu \)M BAF, respectively (\( P < 0.05 \); Fig. 6B). It is worth noting that the decrease in the accumulation of MPP caused by, presumably, the BAF-induced collapse of the ELP pH gradient was similar in size to the slowly exchanging pool of \( {^{3}}\text{H} \)MPP evident in the time course of MPP efflux that followed 20 min of loading (Fig. 3A). In contrast, coexpression of OCT2-expressing cells to 10 \( \mu \)M BAF had no effect on 5-min accumulation of \( {^{3}}\text{H} \)MPP (Fig. 7).

BAF is a macrolide antibiotic, and, as a nonelectrolyte with a molecular mass of 683 Da, it appears unlikely to interact directly with MATE1. Nevertheless, the neutral sterol corticosterone has been shown to inhibit MATE1 activity (23), so we examined the direct effect of BAF on the initial rate of uptake of \( {^{3}}\text{H} \)MPP and \( {^{3}}\text{H} \)NBD-MTMA. As shown in Fig. 8, acute exposure of MATE1-expressing cells to 1, 5, or 10 \( \mu \)M BAF had no effect on transport of either substrate through 5 min. By 10 min, however, 5 and 10 \( \mu \)M BAF began to reduce accumulation of both substrates, and all three concentrations reduced accumulation by 15 min. These data show that the BAF-induced reduction of MPP and NBD-MTMA accumulation in MATE1-expressing cells shown in Fig. 6 did not reflect a direct effect of BAF on the transporter and support the view that the decrease in OC uptake at 15 min was a consequence of inhibiting the secondary accumulation of OC within the acidic ELP compartment.

The slowly exchanging pool of MPP evident in the time course of efflux from cells that express MATE1 (Fig. 3)
presumably reflected sequestration of MPP within the acidic ELP. Figure 9 shows a comparison of the time course of MATE1-mediated efflux of $^{[3]H}$MPP from cells preloaded with the (30 nM) substrate for 20 min with that determined in cells that were incubated for 10 min in the presence of 10 μM BAF before being loaded for 20 min with labeled MPP (plus continued exposure to 10 μM BAF). In four separate experiments, the plateau level was reduced from 35.4% of time 0 MPP content in control cells to 9.8% (±3.7%) in BAF-treated cells ($P < 0.05$), consistent with the conclusion that the plateau level reflected sequestration of substrate within acidic elements of the ELP. Interestingly, the rate constant for efflux from the “fast compartment” (presumably the cytoplasm) was not changed by BAF exposure (1.6 ± 0.18 vs. 2.0 ± 0.28%/s for control and BAF-treated conditions, respectively, $P > 0.05$), suggesting that the effect of BAF was simply to eliminate sequestration and leave efflux from the rapidly exchangeable cytoplasmic compartment unchanged.

The evidence described to this point concerning the influence of MATE1 on intracellular sequestration of OC has been limited to cultured cell data. It was of interest to extend these observations to the distribution of OCs within intact renal tubules. We have previously shown (42) that rabbit RPTs express Mate1 (and Mate2-K). Figure 10 shows the distribution of NBD-MTMA in an intact nonperfused rabbit renal tubule loaded with 50 μM NBD-MTMA for 20 min at 37°C. Indeed, the distribution of the fluorescent compound included a punctuate signal as well as a more uniform distribution through the cytoplasm. This observation suggested that the expression of MATE protein in native renal tubules also results in subcellular sequestration of a fraction of accumulated OCs. To quantify this effect, we measured the influence of BAF on the accumulation of radiolabeled MPP and NBD-MTMA in nonperfused renal tubules. Figure 11 shows that coexposure of tubules to these substrates plus 5 μM BAF significantly reduced 10-min accumulation of both substrates (31% and 35% for MPP and NBD-MTMA, respectively, $P < 0.05$), similar to the effect of BAF noted for uptake into MATE1-expressing CHO cells (Fig. 6).

In separate experiments, we measured the effect of BAF on OC secretion using perfused rabbit RPTs. Figure 12A shows that exposure of tubules to 5 μM BAF, a concentration that decreased accumulation of MPP by >30%, had no effect on steady-state transtubular secretion of MPP. In contrast, and as expected, coexposure of tubules to $^{[3]H}$MPP plus a 1 mM concentration of the prototypic OC substrate TEA reversibly reduced MPP secretion by ~70% ($P < 0.05$).

**DISCUSSION**

The present results support the hypothesis that MATE1 protein is functionally expressed within membranes of the ELP and that the acidic nature of this intracellular compartment can drive significant accumulation of OCs through the activity of MATE1-mediated OC/H$^+$ exchange. The resulting sequestration of OCs can amount to >30% of the steady-state substrate load in MATE1-expressing cells, including native RPTs. However, exocytosis of OC-laden elements of the ELP does not appear to contribute significantly to steady-state rates of renal OC secretion.

We confirmed the results of our previous report (10) that intracellular accumulation of the fluorescent OC NBD-MTMA in MATE1-expressing CHO cells includes at least two compartments, visually evident as diffuse fluorescence distributed throughout the cytoplasm plus numerous punctate, bright elements distributed throughout the cell (Fig. 4, A and B). The fluorescent substrate associated with these bright elements turns over slowly, as evident from their prolonged presence after the rapid loss of the diffuse cytoplasmic signal that follows the permeabilization by saponin of the plasma membrane (10). Here, we showed that the kinetics of efflux of radiolabeled MPP from MATE1-expressing cells is consistent with the presence within the cytoplasm of a second intracellular compartment that 1) holds >30% of the total steady-state cell load of the substrate and 2) supports a rate of substrate efflux (across the ELP membrane) that is ~10 times slower than efflux from the cytoplasm (across the plasma membrane; Fig. 3).

Three observations support the hypothesis that the intracellular sequestration of OCs in MATE1-expressing cells reflected the presence of functional MATE1 protein within the membranes of acidic elements of the ELP. First, the fluorescence intensity of the punctate sites of NBD-MTMA accumulation appeared to contribute significantly to steady-state rates of renal OC secretion.

Fig. 9. Time course of MPP efflux from hMATE1-expressing CHO cells. After a 20-min preload of ~30 nM $^{[3]H}$MPP or ~30 nM $^{[3]H}$MPP + 10μM BAF, the amount of labeled MPP remaining in cells was measured as a function of time after efflux was initiated in MPP-free buffer of pH 7.4. Each point is the mean $^{[3]H}$MPP content (±SE) determined in four separate experiments, expressed relative to total cell content at time 0. The solid lines represents one-phase exponential decline in cell $^{[3]H}$MPP (rate constant of efflux shown); the dashed lines represent the plateau. All samples were corrected for extra-cellular carryover using $[^{14}C]$mannitol.
Fig. 10. Fluorescent distribution of OCs in intact renal tubules. Shown is a fluorescent image of NBD-MTMA uptake and distribution within an intact nonperfused rabbit renal proximal tubule (RPT; 50 μM; 10-min uptake).

The suggestion that the ELP of RPTs expresses functional OC/H+ exchange is not new; as noted above, Pritchard et al. (25) suggested that the accumulation of OCs by V-type H+-ATPase inhibitor BAF (Fig. 4, A and B vs. E and F). Second, exposure of cells to BAF reduced the steady-state accumulation of radiolabeled MPP and NBD-MTMA by 30% to 40% (Fig. 6). Importantly, this reduction of net substrate accumulation was secondary to the influence of BAF on an intracellular compartment (i.e., the ELP), as shown by the failure of acute exposure to BAF to influence the initial rate of transport of either MPP or NBD-MTMA (Fig. 8). Third, the kinetics of MPP efflux from MATE1-expressing cells that were pretreated with BAF revealed a marked reduction in the size of the slowly exchanging intracellular compartment but no change in the rate of efflux across the plasma membrane (Fig. 9). BAF-sensitive OC accumulation was also observed in intact rabbit RPTs; treatment of tubules with BAF reduced accumulation of MPP and NBD-MTMA by >30% (Fig. 11), similar to the effect noted in CHO cells that heterologously expressed hMATE1. Taken together, these observations support the view that MATE1 is functionally expressed in the membrane of the ELP and that the outwardly directed H+ gradient (developed by V-type H+-ATPase) supports the secondarily active accumulation of MATE1 substrates via OC/H+ exchange.

The present observation of BAF-sensitive OC accumulation in both MATE1-expressing cultured cells and in native renal tubules is consistent with the observations of Pritchard and colleagues (25). It is noteworthy that the influence of BAF was quantitatively similar in both cultured cells that heterologously expressed hMATE1 (Fig. 6) and in native rabbit RPTs (Fig. 11). We assumed that the BAF-sensitive accumulation of OCs in rabbit RPTs reflected the functional expression of rabbit Mate1 (and/or rabbit Mate2-K) within the ELP, as well as in the luminal membrane, of these cells. Three lines of evidence support this assumption, which is predicated on the wide acceptance that MATEs are the functional basis for the OC/H+ activity of the apical membrane of mammalian RPTs (including rabbit RPTs) (23, 38, 41). First, cells of rabbit cortical tissue do express orthologs of both Mate1 and Mate2-K (42). Second, the kinetic and selectivity characteristics of rabbit Mate1 closely match those of the OC/H+ exchange activity expressed in luminal membrane vesicles isolated from the rabbit renal cortex (42). Third, in the species in which renal MATE expression has been histochemically assessed (human and mouse), it is localized to the apical membrane of proximal tubules (18, 21, 23). Taken together, these observations strongly support the contention that luminal OC/H+ exchange activity in rabbit RPTs reflects MATE activity (i.e., some combination of rabbit Mate1 and rabbit Mate2-K). That MATE protein is functionally expressed in the ELP of rabbit RPTs is supported by the observation of OC/H+ exchange (i.e., MATE) activity in rat renal endosomes (25) and the demonstration of a large, robust pool of subapical ELP elements that cycle through endocytosis and exocytosis in rabbit RPTs (7). Collectively, these data support our hypothesis that the BAF-sensitive component of steady-state OC accumulation in rabbit RPTs and, by extension, in mammalian RPTs in general, reflects the presence of functional MATE protein that exists in a steady-state distribution between the ELP and luminal plasma membrane.

Pritchard et al. (25) suggested that the accumulation of OCs within endosomal compartments of RPT cells could 1) reduce the free concentration of OCs within the cytoplasm of RPT cells and/or 2) contribute directly to the rate of transcellular OC secretion. The former suggestion was based on reports of OC accumulation within intact, nonperfused renal tubules to concentrations in total tissue water as much as 100 times that in the surrounding medium (16, 30), far in excess of the 10- to 15-fold levels of accumulation that could be supported by the ~60- to ~70-mV membrane potential of RPT cells that...
presumably drives OCT-mediated electrogenic uniport. It was reasoned that endosomal sequestration of OCs, mediated by OC/H⁺ exchange activity of acidic endosomes, could account, at least in part, for this observation, without necessitating the presence of active OC accumulation by the basolateral membrane; indeed, 30% of total cell OCs could be contained in an endosomal compartment that 1) is capable of accumulating OCs to a level 20 times that in the cytoplasm (supported by an endosomal pH of ~6) and 2) represents only ~1.5% of total cell volume. Pritchard’s second suggestion was supported by observations of steady-state recycling of endosomal membrane between the cytoplasm and luminal membrane of RPT cells (7) and the demonstration that, in some systems, endosomal exocytosis can contribute to apical secretion of sequestered substrates (19, 20). However, the present results cast doubt on the likelihood of both these suggestions in rabbit RPTs.

The primary evidence against the influence of OC sequestration on transtubular secretion is that, despite the observation that treating nonperfused rabbit renal RPT with BAF reduced steady-state accumulation of MPP and NBD-MTMA by >30% (Fig. 11), the same treatment had no effect on the steady-state rate of transtubular MPP secretion measured in isolated single perfused RPTs (Fig. 12). This suggests that the rate of endosomal volume released into the filtrate through the routine recycling of the ELP into the luminal RPT membrane (7) is insufficient to influence the normal rate of OC secretion, despite the high concentration of OC contained within that volume. In retrospect, this result could have been predicted. In rabbit RPTs, although the endosomal compartment does cycle into and out of the apical membrane, it takes ~20 min for the pool of luminal (apical) ELP elements to turn over (7). Thus, even if the 30% load of OCs within RPTs observed in the present study were to be delivered to the lumen every 20 min, it would account for ~1% of the measured rate of MPP secretion.

With respect to the suggestion that MATE-mediated sequestration of OC within the ELP would reduce the cytoplasmic concentration of these compounds, that could only occur if the rate of exocytosis of the ELP compartment influenced the steady-state distribution of OC between the cytoplasm and peritubular/luminal compartments. Although exocytosis of sequestered materials has been suggested to have such an effect in some tissues [e.g., in some cancer cells (26)], if sequestration had that effect in RPT cells, then its inhibition should produce an increase in the cytoplasmic concentration of OCs that, in turn, should result in an increase in tubular OC secretion. Such an effect was not seen. Nevertheless, the accumulation of OCs within the ELP represents a steady-state load of potentially toxic material that, if released to the cytoplasm, could contribute to their nephrotoxicity.

It was our original intent to understand the impact of OC sequestration on efforts to measure MATE1-mediated OC efflux, and the present data permit an initial comparison of MATE1-mediated uptake and efflux. The rate of hMATE1-mediated radiolabeled MPP efflux, expressed as a clearance (data from Fig. 9) was 0.29 μl·cm⁻²·min⁻¹ [assuming a cell volume of 0.3 μl/cm² (10)]. The nominal intracellular concentration of [³H]MPP at time 0 (in the “exchangeable” compartment) in these experiments was ~320 nM, which was an overestimate of the “free” concentration in light of evidence that 10–75% of accumulated OCs are bound to cytoplasmic protein (Ref. 6 and Z. Wilson, unpublished observations). Regardless, these concentrations are well below the Michaelis constant for MATE1-mediated MPP transport and, so, efflux was expected to be, and was (Fig. 3B), a first-order function of time. In the absence of a proton gradient, the rates of OC uptake and efflux should be equal (34), so MATE1-mediated uptake in the CHO cells used in this study, reported as clearance, should be ~0.3 μl·cm⁻²·min⁻¹. The relevant value for MATE1-mediated clearance was determined by measuring the kinetics of uptake at an extracellular pH of 7.4 (the same pH used in the efflux experiments; Fig. 2). The ratio of maximal flux (3.9 pmol·cm⁻²·min⁻¹) divided by apparent Kᵢ (46.6 μM) results in a calculated clearance [“transport efficiency” (31)] of 0.09 μl·cm⁻²·min⁻¹, some 3.3 times lower than anticipated. The basis for this discrepancy is not clear, but the expectation of equal rates of MATE1-mediated influx and efflux is predicated on the absence of a proton gradient across the membrane. There is speculation that plasma membrane Na⁺/H⁺ activity can result in a local concentration of H⁺ at the extracellular face of cells that is 4- to 20-fold greater than that in the bulk medium (2). CHO cells do display vigorous Na⁺/H⁺ exchange activity (29), which could complicate establishing equal H⁺ concentration inside and out of the cell. Consequently, in our experiments, even a small transmembrane H⁺ gradient could result in both elevated rates of efflux.

Fig. 12. Effect of BAF (A) or 1 mM tetraethylammonium (TEA; B) on the secretion of [³H]MPP by single, isolated perfused rabbit RPTs. Tubules were exposed to a bath consisting of rabbit bicarbonate buffer with dextran containing ~100 nM [³H]MPP (A) or ~50 nM (B) and perfused with rabbit bicarbonate buffer (all solutions at pH 7.4 and 37°C). A: collections of perfusate were made at 5, 10, and 15 min (control 1). The bath buffer was changed to [³H]MPP with 5μM BAF, and collections were made at 30, 35 and 40 min. The bath buffer was then changed back to [³H]MPP only, and collections made at time points of 60, 65 and 70 min (control 2). B: collection of the perfusate were made at 5, 10, and 15 min (control 1). The bath buffer was changed to [³H]MPP with 1 mM cold TEA, and collections were made at 30, 35, 40, and 45 min. The bath buffer was then changed back to [³H]MPP only, and collection were made at time points of 60, 65 and 70 min (control 2). Each point is the mean (±SE) of perfusate collections made in three separate experiments.
(because of the influence of even a modest inwardly directed H\(^+\) gradient) and decreased rates of influx (because of the competitive influence of extracellular H\(^+\) concentration on the apparent \(K_v\) for MPP transport). In this light, the general agreement of measured rates of influx and efflux suggests that future efforts to establish the kinetics of MATE1-mediated efflux may nevertheless be expected to produce physiologically relevant data. The present observation that inhibition of V-type H\(^+\)-ATPase produced by BAF largely eliminates the complicating influence of intracellular sequestration of accumulated substrate should facilitate measurement of the kinetics of OC transport mediated by MATE1.

In conclusion, the expression of MATE1 within intracellular elements of the ELP is correlated with uptake of cationic substrates of this transporter into, and sequestration within, the ELP. This includes cultured cells that heterologously express the cloned transporter and RPT cells that natively express MATE1. The intracellular sequestered pool of OCs can exceed 30% of total steady-state OC content in cultured cells and RPT cells. The driving force for this accumulation is the outwardly directed H\(^+\) gradient established by V-type H\(^+\)-ATPase in ELP membranes. The inhibition of V-type H\(^+\)-ATPase by BAF effectively eliminated the intracellular sequestered pool and reduced steady-state cell content by >30%. Although sequestration represents a potentially "toxic" intracellular reservoir of drug in RPT cells, the BAF-sensitive compartment(s) does not recycle into the plasma membrane of RPT cells at rates sufficient to influence OC secretion.

REFERENCES


