

Unstirred Water Layers and the Kinetics of Organic Cation Transport

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ABSTRACT

Purpose Unstirred water layers (UWLs) present an unavoidable complication to the measurement of transport kinetics in cultured cells, and the high rates of transport achieved by overexpressing heterologous transporters exacerbate the UWL effect. This study examined the correlation between measured J_{\max} and K_t values and the effect of manipulating UWL thickness or transport J_{\max} on the accuracy of experimentally determined kinetics of the multidrug transporters, OCT2 and MATE1.

Methods Transport of TEA and MPP was measured in CHO cells that stably expressed human OCT2 or MATE1. UWL thickness was manipulated by vigorous reciprocal shaking. Several methods were used to manipulate maximal transport rates.

Results Vigorous stirring stimulated uptake of OCT2-mediated transport by decreasing apparent K_t (K_{tapp}) values. Systematic reduction in transport rates was correlated with reduction in K_{tapp} values. The slope of these relationships indicated a 1500 μm UWL in multiwell plates. Reducing the influence of UWLs (by decreasing either their thickness or the J_{\max} of substrate transport) reduced K_{tapp} by 2-fold to >10-fold.

Conclusions Failure to take into account the presence of UWLs in experiments using cultured cells to measure transport kinetics can result in significant underestimates of the apparent affinity of multidrug transporters for substrates.

KEY WORDS kinetics · organic cation · transport · unstirred water layers

ABBREVIATIONS

CHO	Chinese hamster ovary
DDI	Drug-drug interaction
HEK-293	Human embryonic kidney 293
hMATE1	human Multidrug And Toxin Extruder 1
hOCT2	human Organic Cation Transporter 2
J_{\max}	Maximal rate of transport
K_t	Michaelis constant
K_{tapp}	Apparent Michaelis constant
MDCK	Madin-Darby canine kidney
MPP	1-Methyl-4-phenylpyridinium
NBD-MTMA	N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium
TEA	Tetraethylammonium
UWL	Unstirred water layer

INTRODUCTION

The kinetics of substrate uptake into cells can provide context for understanding the role of a transporter in cellular or systemic physiology/pharmacology, as well as insight into the transport mechanism itself. Certainly the measurement of transport kinetics is a central element in the physiological characterization of the process. Furthermore, in recent years the kinetics of ligand interaction with multidrug transporters (i.e., the relationship between ligand concentration and activity of the transporter) has been used to identify potential candidates for unwanted drug-drug interactions (DDIs) and make recommendations concerning the need for further study, including recommendations for conducting clinical DDI studies (1). However, the interpretation of the kinetic parameters of

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transport, including the maximal rate of substrate transport (J_{\max}), the substrate concentration that results in half-maximal transport (K_t), and the concentration of inhibitor that reduces transport by 50% (IC_{50}), relies on confidence in their accuracy.

Cultured mammalian cells, including HEK 293, CHO, MDCK, and CaCo-2 cells, are widely used as platforms to study the activity of cloned, heterologous transport proteins, especially the study of the kinetics of inhibitory ligand interaction with pharmacologically important transporters (e.g., (2,3)). These systems offer a comparatively straightforward means to focus analytical attention on one (or more) experimentally defined processes without the complicating influence of undefined transporters that may contribute to substrate flux in native tissues (see (1)). However, common approaches to the use of such 'over expression' models also carry the risk of introducing experimental artifacts into the measurement of transport kinetics. Our own interest has focused on the application of computational tools to probe the kinetics and selectivity of the Organic Cation Transporters (OCTs) and Multi-drug And Toxin Extruders (MATEs) that play significant roles in the renal clearance of cationic drugs (e.g., (4–8)). While analyzing a data set on the kinetics of OCT2-mediated transport of the prototypical substrate tetraethylammonium (TEA) collected over the course of several studies, we noted a correlation between measured values of J_{\max} and K_t , namely, higher J_{\max} values were routinely associated with higher K_t values (Fig. 1). The K_t , i.e., the apparent 'affinity' of a transporter for substrate, is generally assumed to be an intrinsic property of the transport protein (in that it reflects protein structure). Consequently, we were concerned that the

observed systematic variation in the measured K_t value for TEA's interaction with OCT2 reflected an experimental artifact in its measurement. The data presented here show that the primary explanation for this J_{\max} - K_t correlation is the influence of an unstirred water layer (UWL; also referred to as the aqueous boundary layer) on the measurement of transport kinetics.

That UWLs can introduce error into the measurement of the kinetics of carrier-mediated transport has been recognized since at least the early 1970's (e.g., (9–11)). For carrier-mediated uptake into cells, transport activity reduces the concentration of substrate in the layer of effectively static fluid immediately adjacent to the extracellular face of the membrane, thereby establishing a gradient of substrate concentration between the surface of the membrane and the (well-stirred) bulk medium. Consequently, the concentration of substrate in the experimental solution is greater than that exposed to the transporter, and so 'apparent' K_t values (K_{tapp}) determined experimentally overestimate the actual concentration of substrate required to half-saturate the transporter. To describe the influence of UWLs on the apparent kinetics of carrier-mediated transport, Winne (9) introduced an equation for the substrate concentration in the well-stirred bulk medium that results in half-maximal transport:

$$C = K_t + \frac{0.5 J_{\max} \delta}{D} \quad (1)$$

where J_{\max} is the maximal rate of mediated transport, K_t is the true Michaelis constant of the transporter, δ is the thickness of the UWL above the membrane surface, and D is the diffusion coefficient of the substrate. This concentration corresponds to the apparent Michaelis constant (K_{tapp}):

$$K_{tapp} = K_t + J_{\max} \left(\frac{\delta}{2D} \right) \quad (2)$$

Thus, the bias introduced to experimentally determined K_{tapp} values is proportional to (i) the thickness of the UWL and (ii) the maximal rate of transport. In qualitative terms, at least, a positive correlation between measured values of K_t and J_{\max} , such as that evident in Fig. 1, is an expected consequence of the influence of UWLs on experimentally determined transport kinetics. Indeed, such a correlation between empirically determined J_{\max} and K_t values was recently noted for transepithelial bile salt transport across MDCK monolayers that express the apical bile salt transporter, ASBT (12).

The Winne relationship is based on the assumption that the concentration profile near the membrane is constant with time. In fact, this assumption does not hold for typical uptake studies because achieving that steady state takes longer than

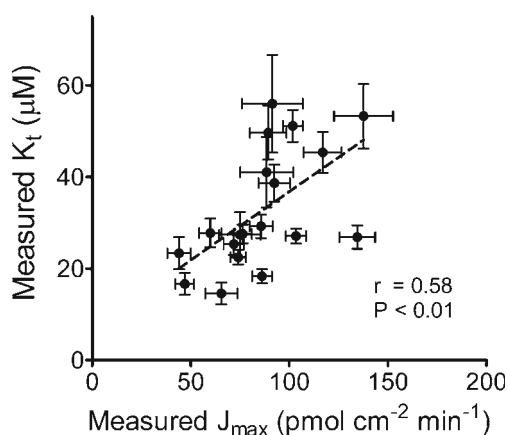


Fig. 1 Relationship between experimentally determined values for J_{\max} and K_t for transport of TEA in CHO cells that stably expressed human OCT2. Each point is the J_{\max} and K_t value as calculated by GraphPad from data obtained in single experiments (of a total of 19) that measured (generally in triplicate in 24 or 48 well plates) [³H]TEA transport as a function of increasing concentration of unlabeled TEA. Error bars indicate SE as determined (by GraphPad) for fitting the Michaelis-Menten equation to the data using nonlinear regression analysis. The dashed line was fit to these data using linear regression.

the course of typical incubation times (a few tens of seconds to minutes). Despite this limitation, the Winne relationship provides a useful basis for assessing the potential influence of UWLs on the experimentally determined kinetics of carrier-mediated transport.

In addition to its dependence on J_{\max} , the impact of UWLs on transport kinetics is a function of the thickness (depth) of the UWL. The depth of UWLs associated with commonly used cell culture plates has been thoroughly examined in the context of their impact on estimates of passive permeability of drugs (13). Without vigorous mixing (of a type difficult to achieve with transport protocols that use cultured cell models), the UWL thickness above the planar surface of a multiwell plate is typically between 1000 and 2000 μm (e.g., (14–17)), making diffusional resistance through this layer a potentially significant element in the measurement of the kinetics of substrate uptake using cultured cells.

In the current study we assessed the quantitative impact of UWLs on the kinetics of substrate transport mediated by human OCT2 and MATE1 expressed in a cultured mammalian cell. The observations suggest that rates of transport commonly achieved in studies using heterologous expression systems can result in overestimates of K_t values by a factor of 2 to 10 or more. Reducing the J_{\max} of transport by, for example, reducing levels of transporter expression, can provide more accurate estimates of K_t .

MATERIALS AND METHODS

Chemicals

[^3H]tetraethylammonium ([^3H]TEA; 54 Ci/mmol) was prepared by American Radiolabeled Chemicals (St. Louis, MO). [^3H]1-methyl-4-phenylpyridinium ([^3H]MPP; 80 Ci/mmol) was prepared by the Synthesis Core of the Southwest Environmental Health Sciences Center/Department of Chemistry of the University of Arizona (Tucson, AZ). The fluorescent organic cation and transported OCT2 substrate (8), N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino]ethanaminium (NBD-MTMA; (18)), was also prepared by the Department of Chemistry of the University of Arizona, as was unlabeled MPP. TEA chloride and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Stable Expression of OCT2

Chinese Hamster Ovary (CHO) cells containing the Flp recombination target site were obtained from Invitrogen (Carlsbad, CA). CHO cells were grown in Ham's F-12 Nutrient Mixture (Sigma-Aldrich) with 10% fetal bovine serum (Fisher Scientific, Pittsburg, PA) and supplemented with 100 $\mu\text{g}/\text{ml}$ Zeocin (Invitrogen). Cells that stably expressed OCT2 or

MATE1 were prepared using methods described previously (19,20). In all the studies presented here the OCT2 protein expressed in CHO cells included the V5 epitope (GKPIPPLLGLDST) added to the C-terminus (immediately following the wild type hOCT2 sequence) or the N-terminus (immediately preceding the hOCT2 sequence). Preliminary experiments showed that the kinetics of MPP transport in both of the constructs did not differ from those measured in cells that expressed the wild type OCT2 sequence without the epitope tag (data not shown). The MATE1 construct used in these studies did not include an added epitope tag. The cells expressing OCT2 or MATE1 were grown and maintained under hygromycin (Invitrogen) pressure (100 $\mu\text{g}/\text{ml}$) in plastic cell culture flasks at 37°C in a humidified atmosphere with 5% CO_2 . Cells were passed every 3–4 days and seeded into 12-well plates (1.1 million cells/well), 24-well plates (550,000 cells/well), 48-well plates (275,000 cells/well), or 96-well plates (110,000 cells/well) as needed, and typically used 24–48 h later.

Measurement of Transport

For studies that used CHO cells grown to confluence in 12, 24 or 48-well plates, the wells were rinsed twice with Waymouth buffer (WB) (135 mM NaCl, 13 mM HEPES, 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM MgCl_2 , 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mM KCl, and 28 mM D-glucose). For experiments measuring OCT2-mediated transport, buffer (room temperature) containing radiolabeled substrate (typically 10–20 nM for [^3H]-labeled compounds), plus the desired concentration of unlabeled substrate, was added individually to each well, removed after a set amount of time, after which the wells were washed three times with cold WB to stop transport. Following the cold WB rinses the cells were solubilized in 200 μl of 0.5 N NaOH with 1% SDS per well and shaken for 15 min. The solubilized cells were neutralized with 100 μL of 1 N HCl and 250 μL were placed in a scintillation vial. The radioactivity in each sample was determined using scintillation spectroscopy (Beckman model LS6000IC). Some studies measured uptake of NBD-MTMA using its native fluorescence (18). Following exposure of cells to WB containing NBD-MTMA (typically 25 μM), accumulated substrate was determined using a fluorescence plate-reader (VarioScan, Thermo) (8). Individual transport experiments were conducted in triplicate and typically repeated at least three times. Experiments were conducted using cells between passages 4 and 35 with no consistent difference in transport rates between earlier and later passages.

Studies that measured transport in cells grown in 96-well plates (Greiner; VWR Intl., Arlington Heights, IL) used an automatic fluid aspirator/dispenser (Model 406, BioTek, Winoski, VT). Plates containing culture media were placed in the unit and automatically rinsed/aspirated three times with

room temperature WB, after which transport buffer (60 μ l) was automatically introduced into each well (the composition and timing of the addition varied based on the experimental protocol; refer to figure legends). In some experiments, following addition of the transport buffer the plate was shaken (reciprocating; 13 Hz) for the duration of the exposure (up to 30 s). In studies measuring MATE1-mediated transport, the cells were first preincubated for 5, 10 or 20 min in 10 or 20 mM NH_4Cl . Transport was initiated by aspirating this medium and replacing it with an NH_4Cl -free medium (thereby rapidly establishing an outwardly-directed H^+ gradient; (21)) containing radiolabeled substrate (w/ or w/o additional ligand), as described for the OCT2 experiments. The transport reaction was stopped by the rapid addition (and simultaneous aspiration) of cold (4°C) WB. Following aspiration of the cold stop, 250 μ l of scintillation cocktail (Microscint 20, Perkin-Elmer, Waltham, MA) was 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, multiwell scintillation counter (Wallac Trilux 1450 Microbeta, Perkin-Elmer). Transport was typically normalized to surface area of the confluent monolayer. For the purpose of comparing transport rates expressed per cm^2 to those in the literature expressed per milligram of cell protein, we have found the conversion factor of 0.035 mg/cm^2 (22) to be reasonably accurate.

shRNA Knockdown of OCT2 Expression

hOCT2-expressing CHO cells and non-expressing wild type CHO cells were seeded in a 96-well plate at a density of 12,000 cells/well. The cells were exposed to lentiviral (LV) transduction particles (multiplicity of infection—MOI—of 2) containing either (i) one of five distinct DNA sequences each coding for a short hairpin RNA sequence designed for the knockdown of hOCT2 (Sigma product SHCLNV-NM_003058); or (ii) an LV transduction particle containing a non-target shRNA sequence (Sigma product SHC002). After 24 h puromycin (60 μ g/ml) was added to the wells to select for cells that stably expressed the LV-introduced shRNA sequence (per manufacturer instructions).

Measurement of RNA

Isolation of total RNA from the cell monolayers and synthesis of cDNA were performed using the Cells-to-cDNA™ II Kit (Applied Biosystems, Grand Island, NY) according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) was performed using the Applied Biosystems 7300 Real-Time PCR System. Each PCR reaction solution was prepared using the TaqMan Gene Expression Assays (Hs00537914_m1) and TaqMan Gene Expression Master Mix according to the manufacturer's protocol (Applied

Biosystems). The PCR was run at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Quantification of relative gene expression was performed using the $\Delta\Delta\text{CT}$ method. The housekeeping gene 18 s rRNA was used for normalization.

SDS-PAGE and Western Blotting

Crude membranes were prepared from OCT2-expressing cells according to Pelis *et al.* (23). Proteins (0.1 μ g/lane) were separated on 8% SDS-PAGE gels and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 1 h in blocking buffer [5% non-fat dry milk in PBS-T (PBS containing 0.05% Tween-20)] at room temperature, followed by overnight incubation (4°C) with mouse anti-V5 antibody (0.1 μ g/ml; Invitrogen) diluted in blocking buffer. After extensive washing with PBS-T, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (0.01 μ g/ml) diluted in blocking buffer. Following extensive washing with PBS-T, the membrane was incubated in SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Protein Biol, Rockford, IL), and the secondary antibody was detected on high performance chemiluminescence film (Amersham ECL; GE Healthcare Bio-Sciences, Pittsburgh, PA).

Statistical Analysis

Data are expressed as means \pm SE, with calculations of standard errors based on the number of replicates within an experiment (typically 3–5), or based on mean values determined in separate experiments using cells at a different passage number. Statistical comparison of differences between sets of experimental observations used two-tailed t-tests (Excel 2007; Microsoft, Redmond, WA); comparison of slopes and intercepts used an extra sum-of-squares F test (Prism 5; GraphPad Software, La Jolla, CA). Observed differences were deemed significant when $P < 0.05$.

RESULTS

Manipulation of δ : The Effect of Shaking on OCT2-Mediated Transport in Cultured CHO Cells

If an UWL is of sufficient depth to influence the rate of carrier-mediated substrate transport, then it follows that reducing its depth should increase substrate uptake. Although the type of stirring/shaking that can be applied to transport experiments with cells cultured in multiwell plates cannot eliminate UWLs (13), it should be possible to reduce their influence. We tested this by assessing the effect of shaking multiwell plates on rates of OCT2-mediated transport of MPP and TEA. The mixing

arising from placing a 24 well culture plate on a flat-bed orbital shaker (GeneMate MP4, BioExpress, Kaysville, UT) rotating at 300 rpm (5 Hz) increased the 30 s uptake of [3 H]TEA (by 16%; $P < 0.05$) and [3 H]MPP (by 50% $P < 0.05$). The more vigorous shaking produced by reciprocal agitation at 13 Hz of a 96 well plate (via the BioTek 406 fluid dispenser), resulted in larger increases in the 30 s accumulation of both TEA (15 nM) and MPP (8 nM) (56 and 72%, respectively; Fig. 2). As a control for the possibility that the mechanical agitation of the cells stimulated the transport process itself (e.g., secondary to activation of mechanically sensitive channels/receptors), we assessed the effect of measuring transport immediately after shaking the cells for 30 s. This treatment had no effect on the subsequent rate of uptake (data not shown), suggesting that the stimulation of transport resulting from agitation reflected a decrease in UWL thickness rather than an intrinsic change in activity of the transporter.

If reduction of the depth of the UWL caused the stimulation of uptake produced by shaking, then the kinetic basis of that stimulation should be a decrease in the apparent Michaelis constant for transport; J_{\max} should remain unchanged (see (24)). This proved to be the case. Figure 3a shows the effect of reciprocal shaking (13 Hz) on the relationship between TEA concentration and the rate of TEA transport into OCT2-expressing CHO cells (corrected for accumulation in wild-type CHO cells). Consistent with the influence

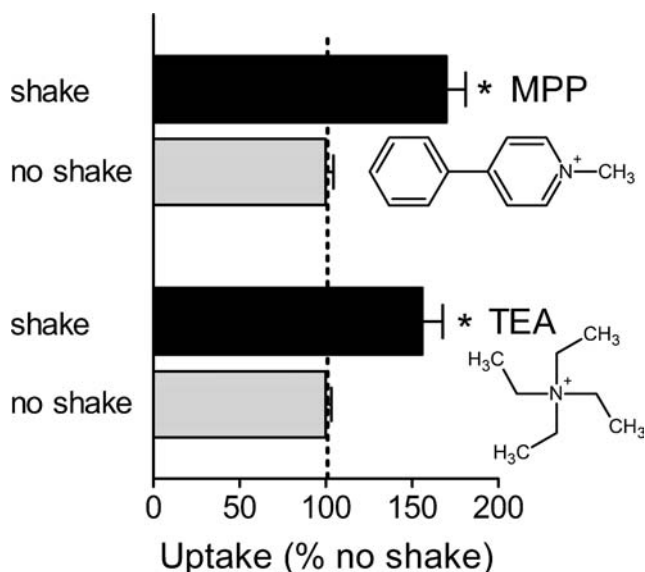


Fig. 2 Effect of shaking on the rate of transport of [3 H]TEA (15 nM) or [3 H]MPP (8 nM) into OCT2-expressing CHO cells. Uptake (30 s) was measured in cells grown in wells of a 96 well plate. Following addition of the medium containing labeled substrate (60 μ l) the plates were either shaken (13 Hz) or left unshaken. The length of each bar represents the average (\pm SE) uptake, normalized to that measured without shaking, determined in three (TEA) or five (MPP) separate experiments (each measured in six wells). Each uptake was corrected for that measured into cells in which mediated transport was blocked by the addition of 1 mM unlabeled MPP. Asterisks (*) indicate differences at the level of $P < 0.05$.

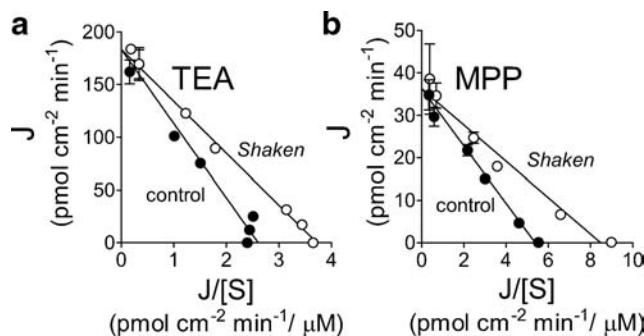


Fig. 3 Effect of shaking (13 Hz) on the kinetics of OCT2-mediated transport of (a) TEA or (b) MPP. In the Eadie-Hofstee plots of the transport data, each point represents the average (\pm SE) of three separate experiments that determined the rate of transport as a function of increasing substrate concentration. In each separate experiment rates of transport at each substrate concentration were determined from 30 s uptakes measured in six wells of a 96 well plate. Open circles show rates of uptake resulting from continuous shaking of the plate following addition of the transport buffer; filled circles show rates of uptake in wells of cells that were shaken for 3 s after addition of the transport buffer and then left unshaken for the remainder of the incubation. OCT2-mediated transport was calculated from total uptake less that occurring by non-saturable pathways (incl. diffusion and non-specific binding).

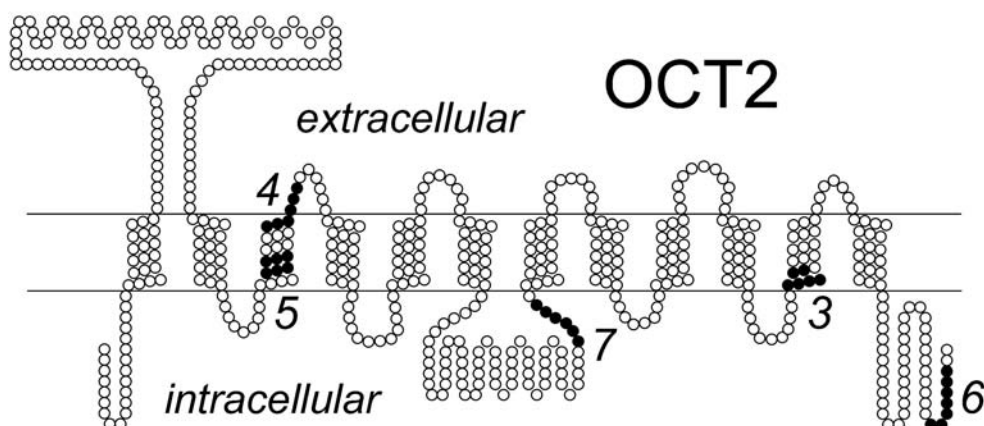
of an UWL on the kinetic profile of TEA transport, in three separate experiments shaking reduced the K_{tapp} of uptake (from 69.5 ± 4.1 to 50.8 ± 2.4 μ M; $P < 0.05$) with no change in J_{\max} (181 ± 7.2 and 188 ± 5.7 $\text{pmol cm}^{-2} \text{min}^{-1}$ for control and shaken, respectively). Shaking produced a similar effect on the kinetics of MPP transport (Fig. 3b), reducing the K_{tapp} of uptake (from 6.5 ± 0.4 to 4.5 ± 0.5 μ M; $n = 3$, $P < 0.05$) with no change in J_{\max} (35.4 ± 1.4 and 37.4 ± 2.5 $\text{pmol cm}^{-2} \text{min}^{-1}$ for control and shaken, respectively).

Effect of Reduced J_{\max} on the Impact of UWL on Transport Kinetics

According to the Winne relationship, the bias introduced to measured values of K_{tapp} is proportional to UWL depth. While shaking can decrease UWL depth, the type of shaking that is practical for use in transport experiments with cultured cells cannot eliminate the UWL. But the bias introduced to K_{tapp} is also proportional to J_{\max} . It follows, therefore, that rather than using an experimental system that maximizes rates of transport (e.g., by maximizing transport protein expression), the kinetics of transport could be more accurately assessed using an experimental system with lower transport rates. To test this hypothesis we used lentivirus-introduced shRNA to knockdown the level of expression of OCT2 in CHO cells that stably expressed the transporter. Of the five shDNA sequences we tested, one (shRNA #6; Fig. 4) was effective in producing a (stable) reduction in OCT2 mRNA ($>90\%$; Fig. 5a), total OCT2 protein (Fig. 5b), and transport function (Fig. 5c). Figure 6 compares the time course of TEA and MPP transport into control cells (OCT2-expressing CHO

Fig. 4 DNA sequence introduced into OCT2-expressing CHO cells, using lentiviral transfection. For each of the five sequences identified in the left hand column with their Sigma-Aldrich lentiviral particle designation the coding sequence found in OCT2 (accession no. NM_003058) is underlined in *bold*. The corresponding portion of the amino acid sequence of OCT2 is also shown, as is the position of that sequence in the proposed secondary structure of OCT2 (25).

Sigma Seq number	OCT2 DNA sequence for shRNA (and associated amino acid sequence)
TRCN0000043243 <i>Sequence 3</i>	CCGGCC CACATTCATTAGGAATCTT CTCGAGAAGATTCCCTAATGAATGTGGGTTTTTG T F I R N L
TRCN0000043244 <i>Sequence 4</i>	CCGGCC CAACCTATACGTGGATGTT CTCGAGAACATCCACGTATAGGTTGGGTTTTTG P T Y T W M L
TRCN0000043245 <i>Sequence 5</i>	CCGGCC TCCTAACTACAGTCTCAT CTCGAGATGAGGACTGTAGTTAGGAGGTTTTTG L L T T V L I
TRCN0000043246 <i>Sequence 6</i>	CCGGCC AAGTTCAGAACTAGACAT CTCGAGATGTCTAGTTTCTGAAC TTGGTTTTTG Q V Q K L D I
TRCN0000043247 <i>Sequence 7</i>	CCGGCC AAGAAATTGAACCTTCAT CTCGAGATGAAGGGTTCAATTTCTTGCTTTTTTG K K L N P S



cells that also stably expressed a ‘non-target’ shRNA sequence; Fig. 5a) and into the ‘knockdown’ cells (cells stably transfected with shDNA sequence #6; Fig. 5b). The reduced level of OCT2 expression in the knockdown cells was associated with 80 and 55% reductions in the 15 s uptake of TEA and MPP, respectively.

We reasoned that the reduced rate of transport supported by the knockdown OCT2 cells should be evident in a reduction in J_{\max} for substrate transport and, consequently, the apparent K_t values for this transport, and this proved to be the case. Figure 7a shows the kinetics of TEA transport into control and OCT2 knockdown cells. In three paired studies, the J_{\max} was reduced by about 90% (from 123 ± 4.4 to 10.8 ± 3.8 pmol $\text{cm}^{-2} \text{min}^{-1}$), and the apparent K_t was reduced from 36.0 ± 4.0 to 17.6 ± 0.2 μM . The OCT2 knockdown also displayed reduced J_{\max} and apparent K_t values for MPP transport (Fig. 7b). In four paired studies the J_{\max} was also reduced by 90% (64.7 ± 16.6 to 6.1 ± 1.2 pmol $\text{cm}^{-2} \text{min}^{-1}$), as expected from the reduction of transporter expression, and the apparent K_t was reduced from 16.6 ± 4.0 to 3.8 ± 0.5 μM .

We also determined the effect on K_{tapp} of increasing J_{\max} by increasing expression of OCT2 in stably transfected cells. Dimethyl sulfoxide (DMSO) has been used as a chemical chaperone to increase membrane expression of mutant proteins (26) and we have used a 24 h exposure to 2% DMSO to produce 2- to 4-fold increases in plasma membrane expression of OCT2 (23)(R Pelis, personal communication). In three experiments the J_{\max} and K_{tapp} for TEA transport in the control

cells exposed to DMSO were 351 ± 84.5 pmol $\text{cm}^{-2} \text{min}^{-1}$ and 87.8 ± 31.9 μM , which were both larger than the values determined in parallel studies with DMSO-exposed OCT2 knockdown cells: 22.0 ± 5.4 pmol $\text{cm}^{-2} \text{min}^{-1}$ and 22.9 ± 2.6 μM .

Figure 8a shows the relationship between J_{\max} and K_{tapp} for OCT2-mediated TEA transport determined in the several experiments ($n=14$) that ‘titrated’ plasma membrane expression of OCT2 as a means to vary J_{\max} . The intercept of this relationship represents an estimate of the true Michaelis constant for OCT2-mediated TEA transport, i.e., the substrate concentration at the membrane at $1/2 J_{\max}$ when the transport capacity is sufficiently low that diffusion through the UWL is not rate-limiting. For OCT2-mediated TEA transport, the estimated true K_t was 11.5 ± 3.8 μM , a value not significantly different from the K_{tapp} for TEA transport measured in the OCT2 knockdown cells (17.6 ± 0.2 μM ; $n=3$). In addition, the same degree of reciprocal shaking that produced a 56% stimulation of transport into the control cells (Fig. 2) had no effect on the rate of OCT2-mediated transport of 17 nM [^3H]TEA into the knockdown cells (data not shown), suggesting that the rate of transport into the knockdown cells was sufficiently reduced to effectively eliminate the influence of UWLs on measurement of K_{tapp} .

Figure 8b shows the relationship between J_{\max} and K_{tapp} for OCT2-mediated MPP transport determined in six paired experiments that measured MPP transport in control and OCT2-knockdown cells ($n=12$). The y-intercept of the

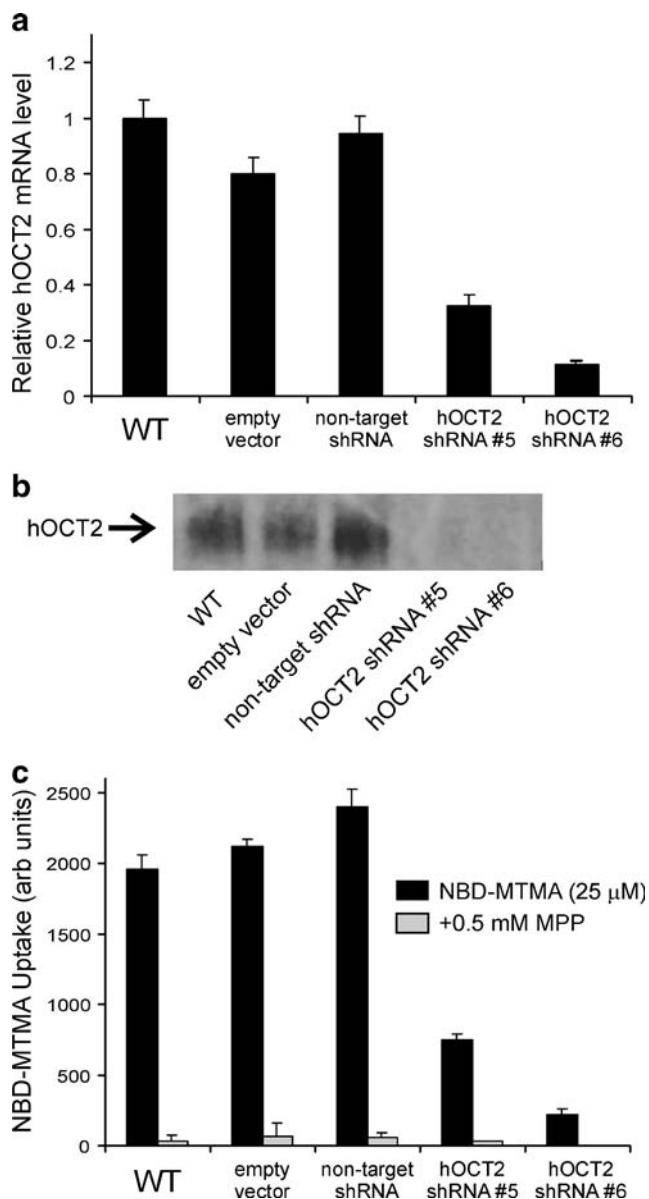


Fig. 5 Effect of lentiviral-introduced shRNAi on the expression of OCT2 mRNA (a), protein (b), and transport function (c). (a) OCT2 mRNA levels (relative to 18s) of CHO cells that stably expressed OCT2 (WT) or OCT2 plus a lentivirus-introduced vector that was either empty; or that generated a non-target shRNA; or one of two shRNAi sequences that targeted OCT2. (b) Western blot of V5-epitope-tagged OCT2 from WT OCT2-expressing cells or cells that stably expressed either the empty lentivirus vector or one of three shRNA sequences (as indicated). (c) Transport of the fluorescent organic cation, NBD-MTMA (expressed in arbitrary fluorescence units) in wild-type OCT2 cells or cells that stably transfected with lentivirus containing one of the indicated sequences. Cells were exposed to 25 μ M NBD-MTMA for 10 min in the absence or presence of 0.5 μ M MPP. The height of each bar represents the average (+SE) of uptake measured in three wells (of a 12 well plate) from a single, representative experiment.

resulting relationship between these parameters was consistent with a true K_t of $1.8 \pm 1.6 \mu\text{M}$, a value not significantly different from the K_{tapp} for MPP transport measured in the OCT2 knockdown cells ($3.3 \pm 0.9 \mu\text{M}$, $n=6$).

According to Eq. 2 the slope of the K_{tapp} vs. J_{max} relationship is proportional to the thickness of the UWL for the experimental system. Assuming a diffusion coefficient for TEA (and all OCs of MW of 150 to 300) of $6 \times 10^{-6} \text{ cm}^2/\text{sec}$ (the measured diffusion coefficient for glucose, MW 180; (27)), the relationship between K_{tapp} and J_{max} observed in all the experiments associated with this study can be accounted for by the influence of an UWL $1656 \pm 147 \mu\text{m}$, which did not differ from the value of $1193 \pm 117 \mu\text{m}$ determined in the experiments with control and knockdown cells. The effective UWL determined from the slope of the K_{tapp} vs. J_{max} relationship for MPP (Fig. 8b) was $1394 \pm 257 \mu\text{m}$, which was not different from the value determined from the compiled TEA kinetic data.

We also examined the effect of reduced rates of transport on the kinetics of transport mediated by a mechanistically distinct process, i.e., the organic cation- H^+ exchanger, MATE1. Figure 9 shows the relationship between J_{max} and apparent K_t determined in seven experiments. J_{max} was varied by altering the outwardly-directed H^+ gradient, which was achieved by preincubating the cells in different concentrations of NH_4Cl for different lengths of time (see figure legend). The slope of this relationship was consistent with an unstirred layer of $1186 \pm 149 \mu\text{m}$, and the intercept suggested a true K_t for MATE-mediated MPP uptake of $7.1 \pm 3.3 \mu\text{M}$.

DISCUSSION

The substrate concentrations that determine rates of transport are those immediately adjacent to the membrane, at the interface between the membrane and the overlying solution. Above the membrane is an unstirred water layer (UWL); operationally this is the static water layer immediately adjacent to the membrane and the overlying layers of slow laminar flow that extend to the bulk solution and through which movement of solutes is limited to diffusion (28). As noted by Barry and Diamond (24), the UWL results in concentrations at the membrane that can differ from that in the bulk solution by 10-fold or more. For transport processes that support net substrate entry across the plasma membrane, the inevitable consequence of an UWL is a substrate concentration at the membrane that is less than that in the overlying, well-stirred bulk solution. It follows that empirically determined kinetic 'constants,' including values for K_t , overestimate the 'true' values reflective of substrate (or inhibitor) concentrations at the membrane. In some instances, the extent of the overestimation may be negligible for all practical purposes. The data presented here, however, show that commonly used approaches for measuring transport activity of cloned transport proteins heterologously expressed in cultured cells can overestimate K_t values by 2 to 10-fold or more.

Winne's theoretical assessment of the impact of UWLs on measurement of Michaelis constants (9) (see Eq. 1) identified

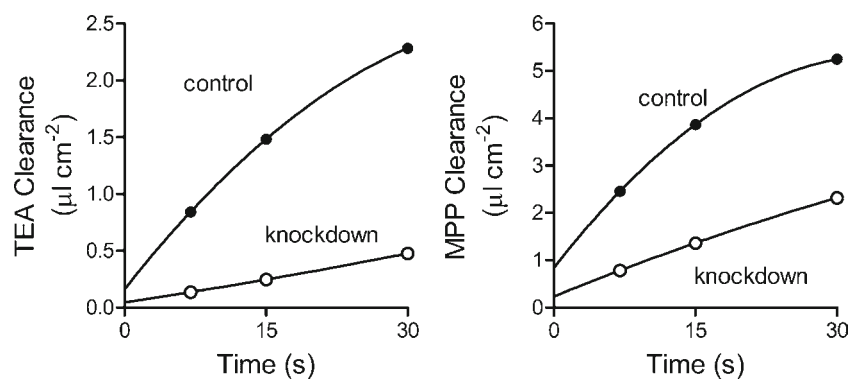


Fig. 6 Time course of uptake of OCT2-mediated MPP and TEA in CHO cells stably transfected with either the empty lentiviral vector (control) or shRNAi sequence #6 (knockdown; see Figs. 4 and 5). Uptakes of the two substrates were measured during simultaneous exposures to the two substrate ($1.3 \mu\text{M}$ [^{14}C]TEA plus 1.0 nM [^3H]MPP). Each point is the average (SE) of uptake measured in triplicate wells of a 12 well plate and expressed as clearance of substrate from the transport buffer. Results shown are from a single representative experiment.

two parameters that functionally define the extent to which transport constants are exaggerated: (i) the operational thickness of the UWL, or δ ; and (ii) the rapidity with which substrate can be removed from the fluid at the membrane, or J_{max} . The factors that influence δ in experimental systems have been examined extensively (13,24,29), but two points are worth emphasizing here. First, in the multiwell cultured cell systems most commonly used to study rates of transport supported by cloned proteins (30), without (very) vigorous stirring, UWLs are 1000 to 2000 μm in thickness (13). Second, even vigorous stirring (using orbital or reciprocal shaking) cannot eliminate UWLs (13); the most vigorous stirring we were able to use still left UWLs that were likely to have been 500–1000 μm in thickness. Nevertheless, the modest stimulation of OCT2-mediated transport of TEA and MPP that was produced by shaking (Fig. 2) was accounted for by decreases in the apparent Michaelis constants for this transport, rather than by changes in J_{max} for either substrate (Fig. 3), and UWLs influence the apparent K_t , not the J_{max} , of a transport process (24). It is also worth noting that the level of stirring/shaking tolerated by our experiments reflected the use of CHO cells

(because they adhere so well to the plastic substrate of culture plates). Unfortunately, HEK 293 cells, which are the most commonly used expression system for studies of OCT- and MATE-mediated transport, frequently lift from the plate upon application of comparatively mild agitation (Morales and Wright, personal observation). Consequently, we suggest that experimental results obtained using cells cultured in unshaken multiwell plates should be interpreted in the light of the likely presence of UWLs that, on average, are on the order of 1200–1500 μm in depth.

The ubiquity of UWLs and the practical inability to substantially reduce them in many experimental situations means that their impact on measurement of transport constants is effectively defined by the J_{max} for substrate transport. The J_{max} of a transport process is the product of transporter turnover number and the number of functional transporters expressed at the plasma membrane. For practical experimental reasons, common practice has been to increase the level of heterologous transporter expression as a means to increase rates of substrate uptake, thereby increasing the transport ‘signal’ compared to the ‘noise’ represented by the level of substrate

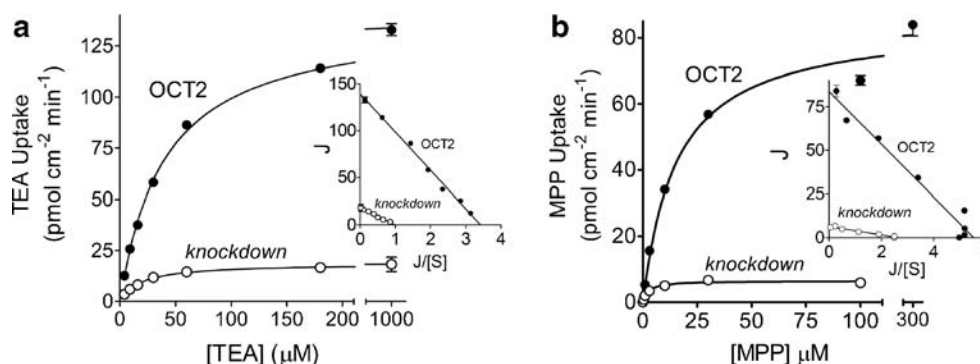


Fig. 7 Kinetics of OCT2-mediated TEA transport (a) or MPP transport (b) in control (fully expressing) cells or in cells from which OCT2 expression was reduced by stable expression of shRNAi sequence #6 (knockdown). Each point is the average (\pm SE) of uptake (30 s) from single experiments in which uptake at each substrate concentration was measured either in triplicate (TEA) or quadruplicate (MPP). For each experiment OCT2-specific uptake was determined by correcting total uptake for that measured in wild type CHO cells. Each curve reflects a non-linear regression fit of the data to the Michaelis-Menten equation. Insets show the Eadie-Hofstee plots for each data set.

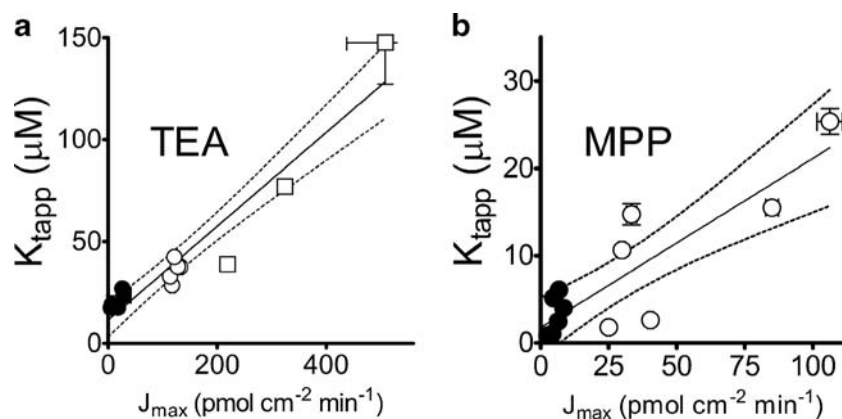


Fig. 8 Relationship between experimentally determined J_{\max} and apparent K_t for OCT2-mediated transport of TEA (a) and MPP (b). Kinetic parameters were determined in a series of experiments using either control OCT2-expressing cells (open symbols) or in cells in which OCT2 expression was reduced through stable transfection of a lentivirus-introduced shRNAi sequence from OCT2 (filled symbols). Square symbols show kinetic parameters determined from experiments in which OCT2 expression was increased by 24 h exposure of cells to 2% DMSO prior to measurement of TEA transport. Each point shows the kinetic parameter values (\pm SE) as determined by non-linear regression analysis (GraphPad). The solid line is a linear regression of J_{\max} vs. K_{tapp} ; dashed lines show the 95% confidence interval (for TEA, $n = 14$; for MPP, $n = 14$).

accumulation observed in cells that do not express the heterologous transport protein. Several methods have been used to maximize expression of heterologous transporter protein in cultured cells, including selection of clonal lines of cells that display the highest rates of transport following transfection and antibiotic selection (e.g., (31)), and induction of protein expression, either selectively (e.g., using zinc-inducible promoters upstream of the transfected transporter sequence; (32)) or, more commonly, through use of sodium butyrate-driven induction of gene expression (33). Indeed, for cells that stably express a heterologous transporter, adding 5 mM

sodium butyrate to the culture media for 24 h prior to experimental use can increase transport activity by 2- to 5-fold or more (34) and is commonly used to increase rates of OCT- and MATE-mediated substrate uptake (e.g., (35,36)). It is, however, ironic that efforts to increase the precision of transport measurements (by increasing J_{\max} values) can compromise the accuracy of the resulting estimates of transport kinetics (by increasing the influence of UWLs on measurement of those same values).

Our examination of the influence of J_{\max} on apparent Michaelis constants for transport mediated by OCT2 and MATE1 focused on two prototypic substrates for these processes, TEA and MPP. TEA was among the first substrates used to characterize OC transport in isolated renal proximal tubules (37) and in membrane vesicles isolated from renal cortex (38,39). TEA was subsequently used to characterize transport by the first cloned OCT (40,41) and MATE (42) transporters. Use of MPP as a test substrate for renal OC transporters followed the observation (43) that it is transported with substantially higher transport efficiency (i.e., ratio of J_{\max}/K_t) than is TEA. MPP is now frequently used as a 'well-transported' substrate for both OCTs and MATEs (22,44). In fact, the increasing use of MPP transport as a measure of activity made it the recommended substrate for use in assays of OCT-based drug-drug interactions in the Food and Drug Administration's 2012 draft Guidance for Industry for Drug Interaction Studies (45). Finally, the structural dissimilarity of TEA and MPP (MCS Tanimoto coefficient of 0.16; see Fig. 2) is correlated with quantitatively distinctive kinetic profiles (Fig. 7), and that provided us with a means to apply a broader test of the influence of UWLs on transport than would a focus on any single substrate.

A decrease in J_{\max} for transport of TEA and MPP should reduce the impact of UWLs on measurement of their respective apparent K_t values. Indeed, according to the Winne

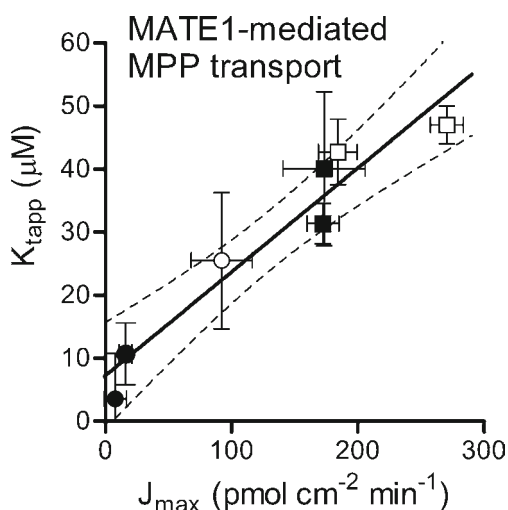


Fig. 9 Relationship between experimentally determined J_{\max} and apparent K_t for MATE1-mediated MPP transport. Kinetic parameters were determined in a series of experiments in which the maximal rate of MPP transport was manipulated by varying intracellular $[\text{H}^+]$ by manipulating the preexposure to NH_4Cl : open squares, 20 min preincubation in 20 mM NH_4Cl ; filled squares, 10 min in 20 mM; open circle, 5 min in 20 mM; filled circles, 10 min in 10 mM. Each point shows the kinetic parameter values (\pm SE) as determined by non-linear regression analysis (GraphPad). The solid line is a linear regression of J_{\max} vs. K_{tapp} ; dashed lines show the 95% confidence interval.

relationship, the degree of bias introduced to measured estimates of an apparent K_t should be directly proportional to the J_{\max} for transport of a given substrate. We decreased the J_{\max} for OCT2-mediated TEA and MPP transport by introducing an RNAi sequence (Fig. 4) that reduced mRNA and translated protein for OCT2 (Fig. 5), and the resulting line of cells with reduced OCT2 expression displayed maximum transport rates for TEA and MPP that were 11-fold lower than those of the 'parental' line of OCT2-expressing cells (Fig. 7). Consistent with the Winne relationship, the decreases in J_{\max} also produced decreases in the apparent K_t values for TEA and MPP (Fig. 8).

The conclusion that the observed changes in K_{tapp} simply reflected the influence of UWLs rather than changes in the transport process itself was ultimately predicated on the assumption that there is, in fact, a unique K_t value for the interaction of a particular substrate molecule with its transporter. In other words, we assume the K_t for interaction of a transport protein with a particular substrate is an intrinsic property of the protein that reflects the protein's structure under a particular set of physiological conditions (as defined by the prevailing relevant forces, including membrane potential, local ionic conditions and the status of any regulatory process that may influence protein structure). Alternatively, increases in expression of OCT2 protein could lead to increased formation of OCT2 oligomers that display reduced affinity for substrate. However, whereas OCTs have been shown to form homomultimers in the plasma membrane (46,47), the K_{tapp} values for OCT-mediated transport of both TEA and MPP are not influenced by oligomerization (46).

Further support for UWLs as the probable explanation for the correlation between J_{\max} and K_{tapp} was the observation that changes in the J_{\max} for MATE1-mediated transport that were independent of the amount of protein in the membrane were also associated with changes in K_{tapp} (Fig. 9). In the experiments using MATE1-expressing cells we varied J_{\max} for MPP transport by taking advantage of the characteristic stimulation of MATE-mediated OC transport produced by increasing intracellular $[H^+]$ (42,48). That stimulation reflects an increase in the J_{\max} of OC/ H^+ exchange brought about by elevation of the *trans* concentration of hydrogen ion (presumably due to an increase in turnover of the transporter; (49)). Systematic variation of the 'ammonia pulse' used to acidify the cytoplasm of MATE1-expressing cells varied the J_{\max} for transport by more than 20-fold, and decreases in J_{\max} were strongly correlated with decreases in K_{tapp} for MPP transport (Fig. 9). Importantly, if the causal link between changes in J_{\max} and K_{tapp} is an UWL that is a common property of the experimental system, then the slope of that relationship should be proportional to the depth of the UWL and, therefore, the same for all transport. Thus it was significant that the slopes of the relationship between J_{\max} and K_{tapp} for transport of the same substrate (MPP) by two processes that have distinct energetic mechanisms (i.e., OCT2 and MATE1; Figs. 8b and 9), did not differ significantly from the slopes that described the

correlation observed for transport of two distinct substrates (TEA and MPP) by the same process (i.e., OCT2; Fig. 8a and b). It is also worth emphasizing that the slopes of the three J_{\max} vs. K_{tapp} relationships (Figs. 8 and 9) did not differ from the average value of 1500 μM previously reported for UWLs in unstirred multiwell culture plates (13,16).

Interpreting the kinetic parameters for OC transport measured here in the context of those reported in the literature is challenging given the substantial degree of variability in the published values (see (44)). It is, however, instructive to examine them in the light of the expectation that UWLs should influence all estimates of the K_{tapp} for transported substrates. Table I lists published values for J_{\max} and apparent K_t for transport of TEA and MPP mediated by human OCT2 expressed in cells (CHO or HEK-293) that stably expressed the transporter and were grown on multiwell culture plates. Table I also lists the calculated Winne bias, in μM , predicted to be included in the K_{tapp} values owing to the influence of the associated J_{\max} and the presence of a 1500 μM UWL. The J_{\max} and K_{tapp} values reported in the present study were within the range of these previously published values. It is noteworthy that there is a marked correlation between reported J_{\max} and K_{tapp} values; the highest and lowest values for K_{tapp} for OCT2-mediated TEA and MPP transport (500 and 20 μM for TEA; 29 and 8 μM for MPP) were associated with the highest and lowest J_{\max} values for transport of these substrates (805 vs. 63 $\text{pmol cm}^{-2} \text{min}^{-1}$ for TEA; 101 vs. 13 $\text{pmol cm}^{-2} \text{min}^{-1}$ for MPP).

As noted earlier, the influence of UWLs on kinetic constants can be small relative to the probable value of the true K_t for transport of a substrate. For example, Table I also lists published values for the kinetics of OCT2-mediated metformin transport. The median J_{\max} for metformin transport ($\sim 350 \text{ pmol cm}^{-2} \text{min}^{-1}$) is substantially greater than that for either TEA or MPP transport (97 and 35 $\text{pmol cm}^{-2} \text{min}^{-1}$, respectively). Consequently, the bias introduced to measured K_{tapp} values for metformin (around 75 μM) should be substantially larger than that introduced to K_{tapp} s for TEA ($\sim 20 \mu\text{M}$) and MPP ($\sim 5 \mu\text{M}$). However, the 'metformin bias' represents only about 7% of the $\sim 1066 \mu\text{M}$ K_{tapp} reported for OCT2-mediated metformin transport, which is negligible in the face of a true K_t of, perhaps, $\sim 1000 \mu\text{M}$. The complicating influence of UWLs is, however, more substantial when dealing with substrates that have transport properties like those of TEA and MPP. The data presented here suggest that the true K_t for OCT2-mediated TEA transport is ~ 10 to 15 μM , so a 20 μM bias is $\geq 150\%$ larger than the actual K_t . The values of $\sim 50 \mu\text{M}$ routinely reported in the literature (Table I), reflect an underestimate of the affinity of OCT2 for TEA of $>300\%$. With respect to MPP, our data suggest a true K_t of ~ 1 to 2 μM , indicating that literature values underestimate the affinity of OCT2 for MPP by 250% to $>700\%$. There are, of course, many potential sources of inter-study variation in the kinetic parameters generated by different groups (see (58)), and it is unlikely that UWLs are the basis

Table 1 Literature Values for Experimentally Determined Kinetic Parameters for OCT2-Mediated Transport of TEA, MPP and Metformin

Substrate	J_{\max} (pmol cm ⁻² min ⁻¹) ^a	K_{tapp} (μM)	Winne 'bias' (μM)	Reference
TEA	805	500	168	(50)
	132	431	28	(51)
	99	54	21	(52)
	97	35	20	Present study
	63	20	13	(19)
MPP	101	29	21	(53)
	88	25	18	(50)
	35	9	7	Present study
	23	9	5	(8)
	20	20	4	(3)
	16	11	3	(5)
	13	8	3	(54)
Metformin	417	1380	87	(55)
	412	3356	86	(3)
	350	1066	73	(56)
	265	378	55	Morales & Wright, unpublished
	67	215	14	(57)

^a J_{\max} values expressed per mg of membrane protein were converted to values expressed per cm² of cell surface area by using the factor 0.035 mg/cm² (22)

of all (or in some cases, perhaps, even most) of the exaggeration of apparent Michaelis constants for OC transport that appears common in the literature. While it is beyond our means to discern all the reasons for the variability in kinetic constants for OC transport evident in the literature (e.g., (44)), the potential influence of UWLs on such measurements can and should be a part of the interpretation of all transport kinetics.

Although UWLs cannot be eliminated from experimental systems commonly used to characterize membrane transport, their influence on the measurement of the kinetic parameters of drug transport can be significantly reduced, thereby increasing the accuracy of experimentally determined Michaelis constants. However, before applying methods to reduce the influence of UWLs on experimental estimates of transport kinetics, we suggest that the Winne relationship be used to obtain an order-of-magnitude estimate of the bias that UWLs may introduce to measured kinetic parameters. It is simple to estimate the potential bias that the presence of an UWL may introduce into an empirically determined K_t : divide the product of the measured J_{\max} (expressed in moles per cm²-sec) and an estimate of the UWL (e.g., 0.15 cm; (13)) by 2D (where D is $\sim 6 \times 10^{-6}$ cm²/sec). The result, when converted to moles per liter from its value in moles/cm³, can be compared to the measured kinetic constant. In the event that UWLs may be introducing unacceptably large error to the estimate, use of vigorous stirring ($\gg 10$ Hz), or cells with limited expression of the transport protein (or both) can significantly reduce the error that UWLs introduce to the measurement of transport kinetics.

CONCLUSION

Heterologous expression systems that use cultured cells grown in multiwell plates offer the means to measure the kinetics of multidrug transport with substantial precision. The accuracy of the resulting values is, however, in question; the physical characteristics of such systems, particularly the ubiquitous presence of unstirred water layers, make them vulnerable to overestimating substrate and inhibitor binding constants. The present study showed that commonly used methods to measure the kinetics of organic cation interaction with OCT2 and MATE1 can result in 2 to 10-fold overestimates of K_t values. The accuracy of these measurements can be improved through vigorous stirring of the experimental system or by substantial reduction in the level of expression of the transport protein. Regardless, the increasing use of *in vitro* assessment of ligand binding interactions in clinical, pharmaceutical and regulatory decision-making underscores the importance of interpreting experimental results fully informed of the issues that may influence their accuracy.

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