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special communication

Method for measuring luminal efflux of fluorescent organic compounds in isolated, perfused renal tubules

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Shuprisha, Apichai, Stephen H. Wright, and William H. Dantzler. Method for measuring luminal efflux of fluorescent organic compounds in isolated, perfused renal tubules. *Am J Physiol Renal Physiol* 279: F960–F964, 2000.—To examine directly in real time the efflux of organic compounds [e.g., organic anions (OAs) such as fluorescein (FL)] across the luminal membrane of isolated, perfused renal tubules during net secretion, we devised an approach utilizing a recently developed epifluorescence microscopy system for continuous monitoring of fluorescence in the collected perfusate. To illustrate this approach, we measured the luminal efflux rate of FL in mineral oil-covered, isolated, perfused S2 segments of rabbit renal proximal tubules. The washout profile of FL showed a deviation from linearity at *time 0* when plotted on a semilog scale, indicating that the luminal efflux of FL was a saturable process. We were able for the first time to determine the kinetic parameters of luminal efflux [FL concentration at one-half maximal FL efflux (K_t^{lumen}) of $\sim 560 \mu\text{M}$ and maximal rate of FL efflux across the luminal membrane ($J_{\text{max}}^{\text{lumen}}$) of $\sim 635 \text{ fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$]. From the present study, we conclude that the transport step for OAs across the luminal membrane of OAs is a carrier-mediated process. This approach will work to measure luminal transport in real time for any secreted organic compound that is sufficiently fluorescent to be measured with commonly available, highly sensitive optical equipment.

transport; kidney; *p*-aminohippurate; fluorescein

AN ORGANIC ANION (OA) transport process in the proximal tubules of vertebrate kidneys is responsible for the excretion of a wide variety of OAs (or weak organic acids that exist as anions at physiological pH), among which are potentially toxic substances, including endogenous metabolic waste products, drugs, and xenobiotics (18, 19). The general process, for which *p*-aminohippurate (PAH) and fluorescein (FL) are prototypes, involves transport

into the cells against an electrochemical gradient at the basolateral membrane and movement from the cells into the lumen down an electrochemical gradient (18). Transport into the cells at the basolateral membrane is a tertiary active process, the final step of which is the transport of an OA into the cells against its electrochemical gradient in exchange for a dicarboxylate (DC) [physiologically, α -ketoglutarate (α -KG)] moving down its electrochemical gradient by means of a recently cloned OA/DC exchanger (17, 25, 31). The outwardly directed gradient for α -KG appears to be maintained through a combination of intracellular metabolism and Na^+ -coupled secondary active uptake of α -KG across the basolateral membrane. This model, first based on studies with renal basolateral membrane vesicles (17, 25), has now been shown to function in intact renal proximal tubules from mammals and reptiles (2, 3, 24, 26, 27, 31, 36).

In contrast to the information now available on the mechanism of basolateral transport, very little is known about the process involved in OA efflux across the luminal membrane during net secretion. This movement is down an electrochemical gradient and must be mediated in some fashion to account for the relatively high apparent permeability of the membrane to these hydrophobic substances (7). Studies with rabbit and pig brush-border membrane vesicles are consistent with the idea that the luminal efflux of OAs involves electrogenic-facilitated diffusion (10, 37), but evidence for saturability of this process is still questionable. Although a diverse group of OA transporters located on the luminal membrane has recently been cloned, sequenced, and partially characterized (13, 21, 22, 35), they appear to interact preferentially with large-molecular-weight anionic conjugates and, therefore, appear unlikely to be responsible for the secretion of PAH, FL, and other low-molecular-weight OAs. As-

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pects of the specificity of luminal PAH efflux from rat proximal tubule cells were examined in an *in vivo* study by Ullrich and Rumrich (34). However, it has not been possible to measure the kinetics of the luminal transport process (for any organic electrolyte transporter) while it is occurring in the intact renal proximal tubule.

To approach the problem of studying a luminal transport process directly in intact renal tubules, we employed a recently developed epifluorescence microscopy system for continuous monitoring of fluorescence in perfusate collected from tubules isolated and perfused *in vitro*. We modified the use of this system to measure directly the time course of the washout of FL (or other fluorescent compounds) across the luminal membrane. We showed that the kinetics of a luminal efflux process could be determined from these washout curves. In the process of illustrating this approach, we also clearly demonstrated for the first time that the luminal efflux of OAs involves a saturable process.

METHODS

Chemicals. Spectral grade FL and neutral tetramethylrhodamine-dextran (40,000 mol wt) were purchased from Molecular Probes (Eugene, OR). Light mineral oil and all other chemicals were purchased from commercial sources and were of the highest purity available.

Solutions. A modified rabbit Ringer solution, used throughout the studies as dissection buffer, superfusion bathing buffer, and perfusing solution, consisted of the following (in mM): 110 NaCl, 25 NaHCO₃, 5 KCl, 2Na₂HPO₄, 1.8 CaCl₂, 1 MgSO₄, 10 sodium acetate, 8.3 D-glucose, 5 L-alanine, 4 lactate, and 0.9 glycine; it was adjusted to pH 7.4 with HCl or NaOH. This solution was gassed continuously with 95% O₂-5% CO₂ to maintain the pH. The bathing medium also contained 3 g/100 ml neutral dextran (40,000 ± 3,000 mol wt) to approximate the plasma protein concentration. The osmolality of the solution was ~290 mosmol/kgH₂O.

Preparation of isolated tubules. New Zealand White rabbits, purchased from Myrtle's Rabbitry (Thompson Station, TN), were killed by intravenous injection of pentobarbital sodium. The kidneys were flushed via the renal artery with an ice-chilled solution containing 250 mM sucrose and 10 mM HEPES, adjusted to pH 7.4, with Tris base. They were then gently removed and sliced transversely by using a single-edge razor. A kidney slice was placed in a petri dish containing ice-chilled dissection buffer aerated with 95% O₂-5% CO₂. Dissection of tubules from a slice was performed manually from the cortical zone without the aid of enzymatic agents. All dissections were performed at 4°C, but all experiments were performed at 37°C. We used only proximal S2 segments in this study because the S2 segment of the rabbit proximal tubule is the primary site of OA secretion (38).

Perfusion of tubules. The basic *in vitro* perfusion technique used in these studies was the standard one described previously (5, 6), with the collecting pipette modified so that it had a length of uniform diameter that could be positioned parallel to the bottom of the bathing chamber to serve as a flow-through cuvette (27). Briefly, in this process, each isolated tubule was transferred into a custom-made, temperature-controlled chamber with a coverslip as the bottom. Both tubule ends were held in glass micropipettes, and the tubule was perfused at a rate of ~10–15 nl/min through a micropi-

pette with its tip centered in the tubule lumen. The chamber was continuously superfused with bathing medium at ~3 ml/min, and the temperature of the incoming solution was controlled at 37°C (27).

Determination of FL in collected perfusate. This process has been described previously (27). Briefly, the perfusion chamber was mounted on the stage of an inverted microscope (Olympus IMT-2) fitted with epifluorescence optics. A ×60 oil-immersion objective (1.4 numerical aperture, Olympus) was used to focus excitation light from a 100-W mercury arc lamp and to collect fluorescence emitted from the solution in the collecting pipette. The intensity of excitation light was reduced by a 2.0 neutral-density filter (Oriel, Stratford, CT). FL was excited at 490 ± 10 nm by using a band-pass filter (Oriel) for this wavelength. The excitation light was reflected to the sample with a 490 DRLP dichroic filter (Omega Optical, Brattleboro, VT) which passed >90% of emitted light above 505 nm. The emission fluorescence was first limited to an area of 50 μm diameter by an iris diaphragm. The emission beam was filtered by using a 520 ± 10-nm band-pass filter (Oriel) and counted simultaneously by a photomultiplier tube (Hamamatsu model HC 120–03; Bridgewater, NJ) in photon-counting mode. The fluorescence intensity was integrated at 1-s intervals and saved for subsequent analysis with a MSC II data-acquisition microcomputer interface and software purchased from Oxford Instrument (Oak Ridge, TN). Concentrations of FL were determined from a standard curve constructed at the end of each experiment by retrograde infusion of known concentrations of FL into the collecting pipette, while the bathing solution contained either bathing solution only or bathing solution plus the appropriate concentration of FL (27). The background fluorescence of FL in the bathing medium during transport studies was determined by infusing perfusion solution alone into the collecting pipette, while the bathing solution contained the appropriate concentration of FL for that experiment. The autofluorescence and the appropriate FL background counts were subtracted from the counts obtained during net secretion to yield the absolute FL counts in the collecting pipette. The photon count was then converted into concentration from the standard curve.

Measurement of luminal efflux rate of FL. To examine the rate of efflux of FL across the luminal membrane (J_{FL}^{lumen}) as an isolated process, we employed a new method. We first exposed a perfused tubule to 50 μM FL in the bath. FL at this concentration is ~10 times higher than the K_t , where K_t is the FL concentration that produces one-half of the maximal transepithelial secretion rate (J_{max}), for its net transepithelial secretion, as reported previously (27). Therefore, we expected that exposure of a perfused proximal tubule to this concentration of FL in the bath would raise the intracellular concentration of FL enough so that, at steady-state net secretion, the luminal transporters for FL would be saturated or partially saturated. When net secretion had reached steady state, the bathing solution was switched to water-saturated light mineral oil that had been prebubbled with 95% O₂-5% CO₂ and prewarmed to 37°C. Because FL has negligible lipid solubility, covering a tubule with mineral oil effectively limits FL efflux from the tubule cells to that which occurs across the luminal membrane. When we did not wish to limit efflux to that occurring across the luminal membrane only, we replaced the FL-containing bathing medium with FL-free standard bathing medium.

The rate of efflux of FL across the luminal membrane alone, J_{FL}^{lumen} (in fmol·min⁻¹·mm⁻¹), when the tubule was covered with mineral oil was determined from the relationship shown in Eq. 1. This relationship is the same as that

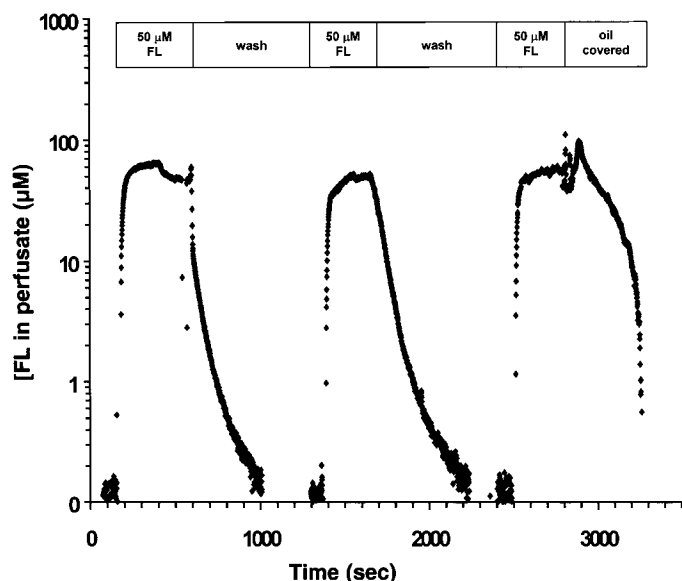


Fig. 1. Sample tracing showing consecutive washout profiles of fluorescein (FL) obtained from a single S2 segment of rabbit renal proximal tubule. The concentration of FL in the collected perfusate is indicated on a log scale on the ordinate. Time is indicated on a linear scale on the abscissa. The type of bathing medium and the presence of FL in the bathing medium are shown in the boxes above the tracing. The tubule was exposed to FL (50 μM) in the standard buffer solution for ~ 5 –8 min until steady-state secretion was reached. The bathing medium was then changed to standard buffer solution without FL (wash) or to prewarmed mineral oil (oil covered).

used for determining net transepithelial FL secretion when FL is present in the standard bathing medium (27)

$$J_{\text{FL}}^{\text{lumen}} = \frac{V_C C_C}{L} \quad (1)$$

In Eq. 1, V_C is the perfusate collection rate (in nl/min) measured directly; C_C is the concentration of FL (in mol/nl) in the collected perfusate; and L is the length of the perfused tubule (in mm) measured with an ocular micrometer. The perfusate collection rate was ~ 10 –15 nl/min. At this collection rate with these solutions, fluid reabsorption is minimal and collection rate and perfusion rate are almost identical (27). As noted previously for net secretion (27), this equation is based on the assumption that there is essentially no reabsorption or backflux of FL from lumen to bath, an assumption shown to hold for PAH (5, 33).

Data presentation. When numerical results are presented to illustrate the method, they are summarized as means \pm SE. The n value is the number of experiments. Each experiment involved a single tubule from a different animal.

RESULTS

Washout profile of FL from isolated perfused S2 segment of rabbit renal proximal tubules. Using our system for continuously monitoring FL fluorescence in collected perfusate (27), we examined the washout of FL from perfused tubules. Figure 1 shows the consecutive washout profiles obtained from a single isolated, perfused S2 segment of rabbit renal proximal tubule. The tubule was exposed to 50 μM FL in the bath until steady-state secretion of FL was achieved (5–8 min). The bathing medium was then switched to either a

FL-free standard solution or mineral oil. The washout using standard buffer solution always yielded a similar profile, confirming that the tubule was still intact after an exposure at high concentration of FL and that the efflux of FL during the subsequent exposure to mineral oil was not an artifact. The semilog plots of the time course of FL concentration in the collected perfusate during exposure to standard FL-free medium in the bath were nonlinear and, significantly, displayed a concave-upward curvature (Fig. 1). This suggested that FL washout under these conditions (which permitted both luminal and peritubular FL efflux from the preloaded tubule cells) was not a simple first-order process but, rather, a multiexponential process. The efflux profile obtained with mineral oil covering the tubule (thereby limiting FL efflux to the luminal membrane) was different (Fig. 1). While also being nonlinear, it revealed a convex-upward curvature consistent with a saturable process (Fig. 1). These data demonstrate directly, for the first time, that the luminal transport step associated with secretion of a low-molecular-weight OA is a saturable process.

Determination of kinetic parameters, K_t^{lumen} and $J_{\text{max}}^{\text{lumen}}$, for the luminal efflux of FL. Figure 2 shows the $J_{\text{FL}}^{\text{lumen}}$ determined from Eq. 1 (see METHODS) as a function of FL concentration inside the tubule cells, $[\text{FL}]_{\text{cell}}$. $[\text{FL}]_{\text{cell}}$ was determined as follows. Knowing the perfusate collection rate (~ 10 –15 nl/min) and the time-dependent concentration profile of FL in the collected perfusate after the tubule was covered with mineral oil, we determined the amount of FL lost from the tubule cells over the time course of the experiment.

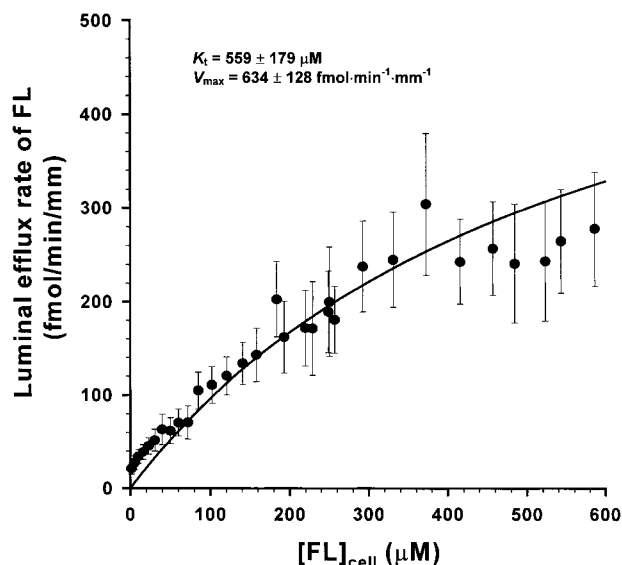


Fig. 2. Concentration-dependent luminal efflux of FL. Each point represents the mean \pm SE ($n = 8$) of luminal efflux rate of FL from oil-covered tubules (shown in $\text{fmol}\cdot\text{min}^{-1}\cdot\text{mm}^{-1}$ on the ordinate) plotted against the changing FL concentration inside the tubule cells at 50 μM intervals (shown in μM on the abscissa). Kinetic parameters, K_t and J_{max} , were derived for each tubule with a nonlinear regression algorithm (Enzfitter, Biosoft) (see RESULTS). Line fitted to data was calculated from Eq. 2 by using averaged kinetic parameters obtained from each individual tubule.

By integrating the amount of FL thus determined over time, and deriving the volume of tubule cell water from the tubule length as described previously (8), we were able to calculate the concentration of FL inside the tubule cells at *time 0* of the washout period. In a similar fashion, we determined the decreasing $[FL]_{\text{cell}}$ during the washout period. Then we determined the $J_{\text{FL}}^{\text{lumen}}$ for each incremental decrease in cellular FL concentration. These data for each tubule were fitted to the following Michaelis-Menten model by using a non-linear regression model (Enzfitter, Biosoft)

$$J_{\text{FL}}^{\text{lumen}} = \frac{J_{\text{max}}^{\text{lumen}} [FL]_{\text{cell}}}{K_t^{\text{lumen}} + [FL]_{\text{cell}}} \quad (2)$$

In Eq. 2, $J_{\text{max}}^{\text{lumen}}$ is the maximal FL efflux across the luminal membrane, $J_{\text{FL}}^{\text{lumen}}$ and $[FL]_{\text{cell}}$ are defined as above, and K_t^{lumen} is the $[FL]_{\text{cell}}$ at one-half $J_{\text{max}}^{\text{lumen}}$. Fitting the data to this relationship, we obtained a mean K_t^{lumen} of 559 ± 179 (8) μM and a mean $J_{\text{max}}^{\text{lumen}}$ of 634 ± 128 (8) $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$.

DISCUSSION

In the present study, we demonstrated a novel technical and analytical approach to real-time measurements of the efflux of fluorescent solutes from cell-to-lumen in intact, isolated perfused renal tubules. To illustrate the utility of this approach, we examined the efflux of FL (as an example of an OA) across the luminal membrane of isolated, perfused S2 segments of rabbit renal proximal tubules. We clearly demonstrated that such efflux involves a saturable and, therefore, apparently a carrier-mediated process. Moreover, using this technique, we were able, for the first time, to determine the kinetic parameters of a luminal OA transporter operating in the physiological context of the intact, perfused tubule. Comparing the luminal transport kinetic parameters (K_t^{lumen} of ~ 560 μM and $J_{\text{max}}^{\text{lumen}}$ of ~ 635 $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$) with those of the basolateral OA/DC exchanger (K_t of ~ 10 μM and a J_{max} of ~ 498 $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$) (29), we see that the luminal OA transporter has a much lower affinity but slightly higher capacity than the basolateral transporter. In conjunction with our previous report of the kinetic parameters of net transepithelial OA secretion (K_t of ~ 4 μM and a J_{max} of ~ 280 $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$) (27), these data support the concept that the OA transport step at the basolateral membrane is rate limiting during net transepithelial secretion.

In summary, we have introduced a novel approach for the measurement of the kinetics of luminal organic solute transport as it occurs in intact, perfused renal tubules. The method is applicable to the determination of the kinetics of the luminal exit of any solute that is sufficiently fluorescent to be measurable by using commonly available, highly sensitive optical equipment. As an example, we showed the efflux kinetics of FL, which is considered a prototype for general OA secretion, although its only proven interaction with a known transporter is with the basolateral OA/DC exchanger (ROAT1) (30). The transporter with which it interacts

during efflux across the luminal membrane has not yet been identified. However, the activities of several other transporters that are suspected of contributing to the renal secretion of different structural classes of OA have been studied by using different fluorescent probes. These include Lucifer yellow [a substrate of ROAT1 and of at least two different secretory processes in the luminal membrane (11)], fluorescein-methotrexate [a suspected substrate of the luminal transporter, OAT-K1 (12)], ochratoxin A [a substrate of ROAT1 (36) and a suspected substrate of OAT-K1 (4)], and fluo 3 [a substrate of the luminal transporter, Mrp2 (16)]. In addition, several fluorescent cationic substrates, including daunomycin (14, 28), quinacrine (15), 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (20, 32), and the novel compound [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium (1), as well as fluorescent derivatives of verapamil (9) and cyclosporin (23), have been used to study organic cation transporters of the kidney and other tissues. All of these fluorescent probes would lend themselves to the study of the kinetics of the luminal efflux step in intact tubules with the approach we have described.

Of course, the use of this technique and the determination of the kinetic parameters are predicated on the assumption that the fluorescent substrate is free (i.e., neither bound nor sequestered) within the cells. We cannot be certain of this for all possible fluorescent substrates. However, in the case of FL, preliminary confocal microscopy data on rabbit S2 segments of proximal tubules under the buffer conditions used in these experiments have provided no evidence of binding or sequestration (S. Shpun and W. H. Dantzler, unpublished observations). In general, it appears that the increasing availability of optically active substrates that can access different renal transport pathways, when used in conjunction with the method described here, offers the promise of substantially increasing our understanding of the transport activity of a heretofore poorly accessible membrane for transport studies: the luminal membrane of renal tubules.

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