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Real-time assessment of α-ketoglutarate effect on organic anion secretion in perfused rabbit proximal tubules

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Shuprisha, Apichai, Ronald M. Lynch, Stephen H. Wright, and William H. Dantzler. Real-time assessment of α-ketoglutarate effect on organic anion secretion in perfused rabbit proximal tubules. *Am. J. Physiol.* 277 (*Renal Physiol.* 46): F513–F523, 1999.—To determine the quantitative roles of the basolateral and luminal Na^+-dicarboxylate (Na-DC) cotransporters in establishing and maintaining the α-ketoglutarate (αKG) gradient required for renal tubular secretion of organic anions, we measured net steady-state transepithelial secretion of fluorescein (FL) in real time in isolated, perfused S2 segments of rabbit renal proximal tubules. Net "basal" FL secretion in the absence of exogenous αKG had a K_t of ~4 µM and a maximal transepithelial secretion rate (J_{max}) of ~380 $fmol \cdot min^{-1} \cdot mm^{-1}$ (where K_t is the FL concentration that produces one-half the J_{max}). It could be almost completely inhibited by basolateral *p*-aminohippurate (PAH). Selective inhibition of the basolateral Na-DC cotransporter indicated that recycling via this transporter of αKG that had been exchanged for FL supports ~25% of the "basal" FL secretion. Physiological αKG concentrations of 10 µM in the bath or 50 µM in the perfusate stimulated net secretion of FL by ~30 or ~20%, respectively. These data indicate that the basolateral Na-DC cotransporter supports ~42% of the net FL secretion. The luminal and basolateral effects of physiological concentrations of αKG were additive, indicating that the combined function of the luminal and basolateral Na-DC cotransporters can support ~50% of the net FL secretion. This apparently occurs by their establishing and maintaining ~50% of the outwardly directed αKG gradient that is responsible for driving basolateral FL/αKG exchange. The remaining ~50% would be maintained by metabolic production of αKG in the cells.

fluorescein; sodium-dicarboxylate cotransporters; transepithelial transport in real time

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A WIDE VARIETY OF ORGANIC anions (or weak organic acids that exist as anions at physiological pH), for which *p*-aminohippurate (PAH) is a prototype, are secreted by the proximal tubules of mammals and most other vertebrates (12, 13). In the S2 segment of mammalian renal proximal tubules, transepithelial secretion of organic anions (OA) 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 (12). Transport into the cells at the basolateral membrane is a tertiary active process, the final step of which is the transport of OA into the cells against its electrochemical gradient in exchange for a dicarboxylate (DC) [physiologically, α-ketoglutarate (αKG)] moving down its electrochemical gradient through an OA/DC exchanger (9, 16). The outwardly directed gradient for αKG appears to be maintained through a combination of intracellular metabolism and Na^+-coupled secondary active uptake of αKG across the basolateral membrane. This basic model, first based on studies with renal basolateral membrane vesicles (BLMV) (9, 16), has now been shown to function in intact renal proximal tubules from mammals and reptiles (1, 2, 17, 24). A transporter that mediates OA/DC exchange has now been cloned from mammalian renal tissue (15, 21).

Most studies that have attempted to determine the role of uptake of exogenous αKG via Na^+-dicarboxylate (Na-DC) cotransport in this OA secretory process have either involved preloading tubules with abnormally high concentrations of αKG to stimulate uptake (2, 19) or have involved nonphysiological buffer solutions and temperature (11). Recently, Welborn et al. (24) used physiological concentrations of αKG (~10 µM) (14) in the bathing medium to examine the role of the basolateral Na-DC cotransporter in establishing and maintaining the outwardly directed αKG gradient for basolateral uptake of OA [using fluorescein (FL)] in isolated renal tubules. Although the medium was as close to physiological as possible in this study, the tubules were not perfused. Therefore, the degree to which basolateral Na-DC cotransport actually functioned to support net transepithelial secretion was still unclear. Moreover, filtered αKG is also reabsorbed by a luminal Na-DC cotransporter, which has been cloned and sequenced (8) and shows a higher capacity than the basolateral Na-DC cotransporter (27). It appeared possible that filtered αKG taken up from the lumen by this transporter also could contribute to the outwardly directed αKG gradient for the basolateral uptake of OA. Indeed, in a previous study with perfused rabbit tubules, we demonstrated that the addition of αKG to the lumen could increase net transepithelial PAH secretion (5). However, this only occurred with abnormally high concentrations of αKG in the lumen and with reduced PAH secretion in the absence of bicarbonate (5). Therefore, we were not certain that this process was of physiological significance.

To determine more rigorously the roles of the luminal and basolateral Na-DC cotransporters in establishing and maintaining net transepithelial secretion of OA, we developed a system whereby we could measure the net steady-state transepithelial secretion of FL in isolated, perfused renal tubules in real time. Using S2 segments of rabbit renal proximal tubules, we demonstrated that such secretion of FL was a saturable, inhibitable process that occurred via the classic OA

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(PAH) transport pathway. We also examined and quantified the roles of the luminal and basolateral Na-DC cotransporters in this transepithelial process under conditions as close to physiological as possible. The results clearly indicate that the basolateral Na-DC cotransporter plays a significant role in recycling αKG at that membrane even in the absence of exogenous αKG. Although the luminal cotransporter appeared to be markedly less important than the basolateral cotransporter, the data indicate that it, as well as the basolateral Na-DC cotransporter, can contribute to basolateral uptake and net transepithelial secretion of FL under physiological conditions when appropriate levels of exogenous αKG are present in both the perfusate and bathing medium.

METHODS

Chemicals. Spectral-grade FL and neutral tetramethylrhodamine dextran (TMRD) (40,000 mol wt) were purchased from Molecular Probes (Eugene, OR). 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 (unless otherwise indicated) as dissection buffer, superfusion bathing buffer, and perfusing solution, consisted of the following (in mM): 110 NaCl, 25 $NaHCO_3$, 5 KCl, 2 Na_2HPO_4, 1.8 $CaCl_2$, 1 $MgSO_4$, 10 sodium acetate, 8.3 D-glucose, 5 L-alanine, 4 lactate, and 0.9 glycine; and was adjusted to pH 7.4 with HCl or NaOH. This solution was gassed continuously with 95% O_2-5% CO_2 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 osmolarity of the solution was ~290 mosmol/kgH_2O.

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 using a single-edge razor. A kidney slice was placed in a petri dish containing ice-chilled dissection buffer aerated with 95% O_2-5% CO_2. 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 (e.g., PAH) secretion (25).

Perfusion of tubules. The in vitro perfusion technique used in these studies was the same as that described previously (3, 4) with some modification so that the collecting pipette 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 (Fig. 1). The outside diameter of the collecting pipette was ~120 μm, and the inside diameter was ~100 μm. The design of the collecting pipette reduced background fluorescence from the bath, which was caused by the addition of FL during transepithelial secretion studies, sufficiently to permit a simple correction. 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 through a micropipette with its tip centered in the tubule lumen at a rate of ~10–15 nl/min. The chamber was continuously superfused with bathing medium at ~3 ml/min and the temperature of the incoming solution was controlled at 37°C as described previously (24). During perfusion experiments, FL was added to the superfusion bathing media and TMRD was added to the perfusion solution as a volume marker.

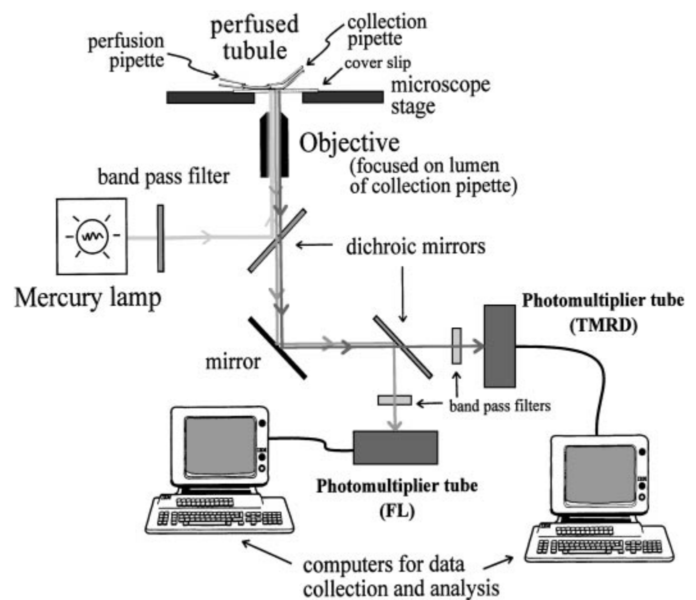

Fig. 1. Instrumentation for real-time measurement of transepithelial secretion of fluorescein (FL). TMRD, tetramethylrhodamine dextran. See METHODS for details.

Measurement of FL and TMRD in collected perfusate. Figure 1 shows the instrument setup diagrammatically. The perfusion chamber was mounted on the stage of an inverted microscope (Olympus model 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 (Oreil, Stratford, CT). Both FL and TMRD were excited at 490 ± 10 nm using a selective band-pass filter (Oriel) for this wavelength. The excitation light was reflected to the sample with a 490DRLP dichroic filter (Omega Optical, Brattleboro, VT), which passed more than 90% of emitted light above 505 nm. The emission fluorescence was first limited to an area of 50 μm diameter by an iris diaphragm and then separated into two beams by a second dichroic mirror (540DRLP, Omega Optical). Each beam was appropriately filtered (520 ± 10 nm for FL; 580 ± 30 nm for TMRD; Oriel and Omega Optical, respectively), and the two beams were simultaneously counted, each by a separate photomultiplier tube (model HC120; Hamamatsu, 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). Figure 2*A* shows a fluorescence profile of FL and TMRD during transepithelial secretion (in arbitrary units) by an S2 segment of proximal tubule after the bathing medium was changed to one containing 250 nM FL as indicated. At the beginning of the experiment (Fig. 2*A*), 30 mg/100 ml TMRD was added to the perfusion solution as a volume marker without any interference with the signal in the FL-detecting channel. During transepithelial secretion of FL, however, fluorescence from FL interfered with fluorescence from TMRD in the TMRD-detecting channel. The

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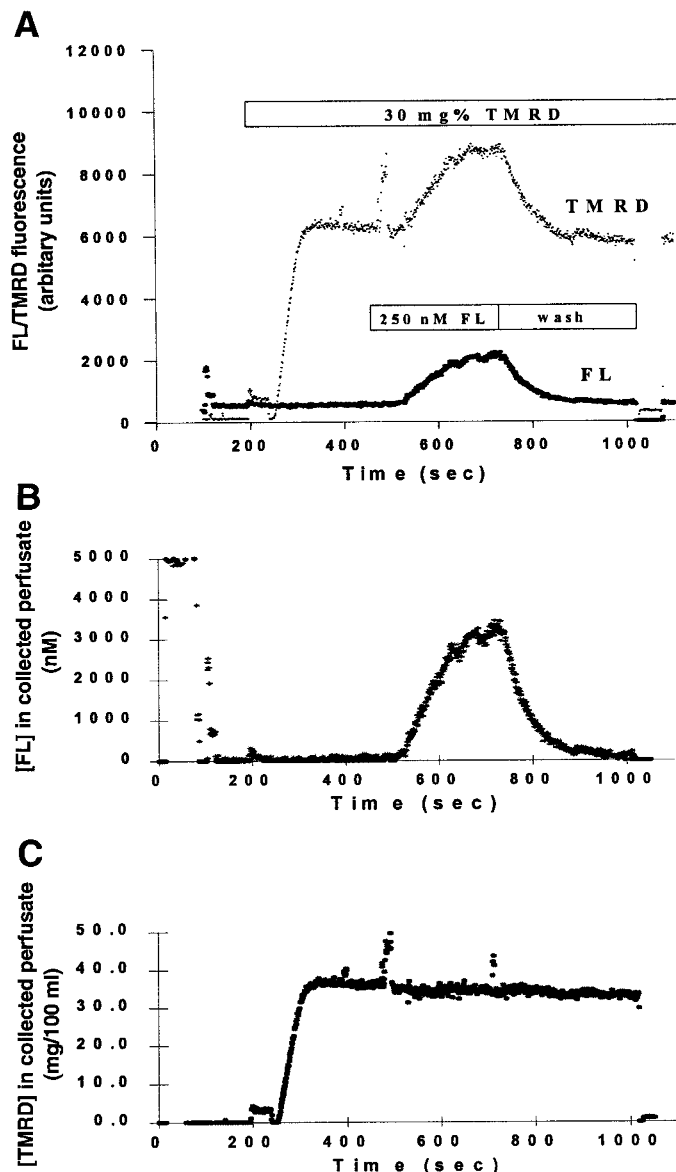

Fig. 2. *A*: fluorescence profiles for FL and TMRD recorded simultaneously from the center of a pipette collecting fluid from an isolated perfused S2 segment of rabbit proximal tubules during a transepithelial secretion study. At the beginning of the experiment, TMRD (30 mg/100 ml) was added to the perfusion solution as a volume marker. This resulted in fluorescence in the TMRD channel without any detectable counts in the FL channel. When the bathing medium was switched to one containing 250 nM FL as well as TMRD, there was a small and rapid increase in background fluorescence within 30 s (not clearly seen in the tracings) followed by an increase in fluorescence from FL that was secreted into the perfused lumen. FL fluorescence was detected in both channels. The larger signal for FL in the TMRD channel than in the FL channel reflects the higher gain in the TMRD channel required to detect the TMRD signal. However, a correction for the contribution of FL fluorescence to the TMRD signal can be made (see METHODS), thereby providing the actual counts of TMRD in collected perfusate. *B* and *C*: actual concentrations of FL (*B*) and TMRD (*C*) in collected perfusate. These were obtained after the original counts in the collected perfusate from each channel were corrected and converted using standard curves constructed at the end of the experiment (all settings and the position of the collection pipette during generation of the standard curves were the same as those during the experiment) (see METHODS).

amount of this interference of FL in the TMRD-detecting channel was determined from standard curves (see below) and simply subtracted to obtain the actual counts for TMRD in the collected perfusate.

Determination of FL and TMRD concentrations in collected perfusate. Concentrations of FL and TMRD were determined from standard curves constructed at the end of each experiment by retrograde infusion of known concentrations of FL or TMRD into the collecting pipette while the bathing solution contained either only bathing solution or bathing solution plus the appropriate concentration of FL. 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 background reading usually averaged less than 1% of total fluorescence during secretion studies. To construct standard curves, we averaged twenty 1-s data points for each FL or TMRD concentration. The interference of the FL fluorescence in the TMRD-detecting channel was also determined during infusion of FL into the collecting pipette. The autofluorescence and the appropriate FL background counts were subtracted from the counts obtained during net secretion to yield the actual FL or TMRD counts in the collecting pipette. The photon count was then converted into concentration from the standard curves. Figure 2, *B* and *C*, shows profiles of the FL and TMRD signals from Fig. 2*A* after conversion to concentrations. About 30 s after adding 250 nM FL to the bathing medium, fluorescence began to rise and reached a steady state within about 5 min. The concentration of FL in the collected perfusate was ~16 times higher than the concentration in the bath indicating transepithelial transport against a concentration gradient. The concentration of TMRD did not change much in this and subsequent studies. Therefore, no measurements of volume change due to water reabsorption were made, and TMRD was used simply as an indicator of leaks in the tubule throughout the study.

Measurement of transepithelial secretion of FL. The net transepithelial secretion of FL, J_{FL} (in mol·min^{-1}·mm^{-1}), was determined from the following relationship

$$J_{FL} = \frac{V_C C_C}{L}$$

In the equation, V_C is the perfusion rate (in nl/min) measured directly; C_C is the concentration of FL (in mol/nl) in the collected perfusate in the collecting pipette during steady-state net secretion; and L is the length of the perfused tubule (in mm) measured with an ocular micrometer. This equation is based on the assumption that there is essentially no backflux of FL from lumen to bath, an assumption shown to hold for PAH (4, 22). The perfusion rate was ~10–15 nl/min. Following the addition of FL to the bath, its concentration at steady state was determined by a 1-min average of data points.

Statistical analysis. Results are summarized as means ± SE; *n* is the number of experiments. One tubule from one animal was used for each experiment. Replicates for each experiment in a single tubule were averaged to represent a single value for that experiment. Differences in steady-state transepithelial secretion rates were evaluated by either a paired *t*-test, a one-way, two-sample *t*-test, or an ANOVA followed by a multiple contrast posttest employing the Dunnett method as indicated in the legends to Figs. 1–9. Differences were assumed to be significant when $P < 0.05$.

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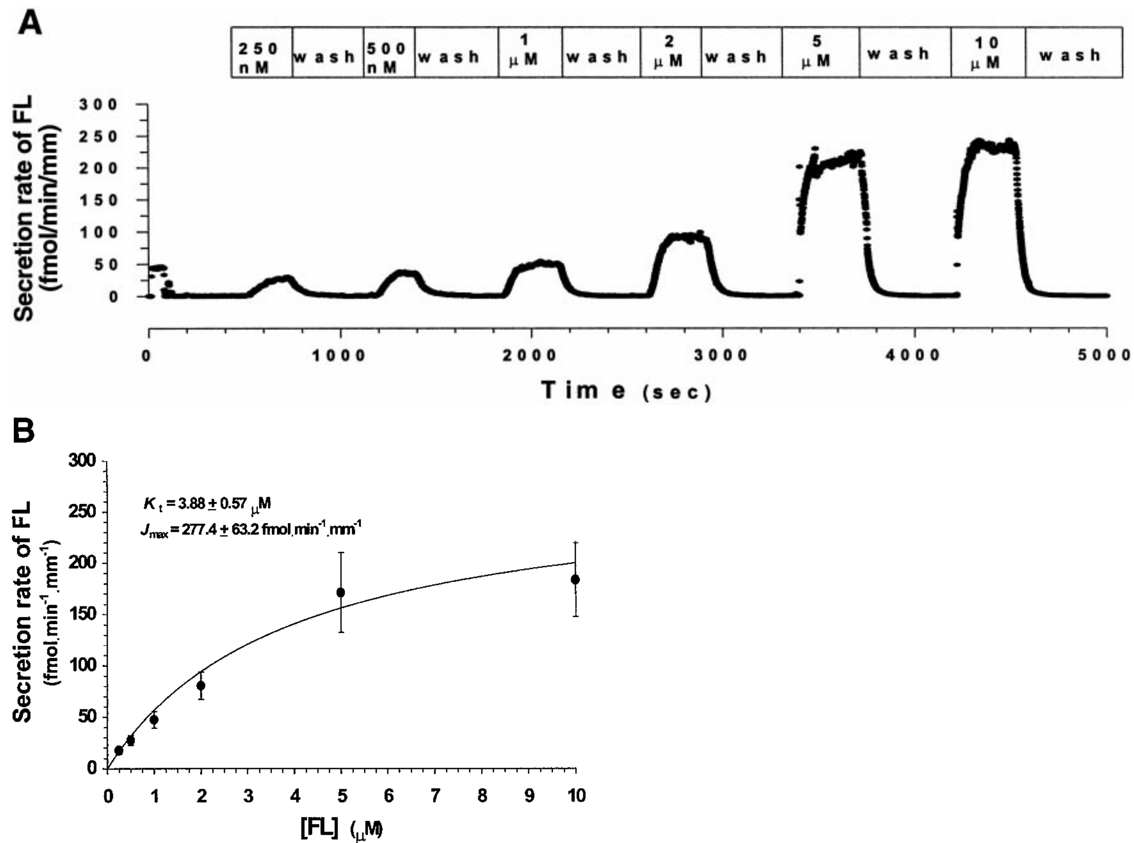

Fig. 3. *A*: a sample tracing of FL transepithelial secretion rate profile in fmol·min^{-1}·mm^{-1} from an isolated perfused S2 segment of rabbit proximal tubules in response to addition of various concentrations of FL to the bath. Tracing was obtained after conversion of concentration profile (as in Fig. 2) using perfusion rate and length of the tubule measured directly in the experiment. A steady-state transepithelial secretion was reached (usually in ~5 min) after FL was added. Then the bathing medium was switched back to standard buffer containing no FL (wash). *B*: effect of increasing FL concentration (shown in μM on abscissa) on rate of transepithelial secretion by perfused S2 segments of rabbit renal proximal tubules (shown in fmol·min^{-1}·mm^{-1} on ordinate). Each point represents mean ± SE (n = 10). Kinetic parameters, K_t and J_{max}, were derived for each tubule with a nonlinear regression algorithm (Enzfitter, Biosoft). Line fitted to data was calculated from *Eq. 1* using averaged kinetic parameters obtained from each tubule.

RESULTS

Kinetics of steady-state transepithelial secretion by isolated perfused S2 segment of renal proximal tubules. Initially, we examined the profile of steady-state transepithelial secretion of FL and determined kinetic parameters of the transport process by S2 segment of proximal tubules. Figure 3 represents a profile of transepithelial secretion by an isolated perfused S2 segment that was incubated in a continuously flowing bathing medium that was alternately switched from a FL-free medium to one containing FL at concentrations ranging from 250 nM to 10 μM. For each concentration of FL, a steady-state secretion of FL was reached ~5 min after the FL was added. This steady-state secretion of FL increased rapidly as the FL concentration increased from 0.25 to 2 μM and then leveled off at higher concentrations (Fig. 3), a typical characteristic of a saturating transport process. Table 1 summarizes the transport rate of FL at different concentrations by isolated perfused S2 segments of proximal tubules in which the perfusion rate was held constant for each tubule but varied from 10–15 nl/min among this set of experiments. Also shown in this table is the tubular fluid-to-bath ratio (TF/B) of 1-mm tubule length (n = 10). This ratio is greater than unity at all concentrations of FL added to the bath, indicating that transepithelial secretion involves transport against a concentration gradient. This ratio decreased gradually from ~5 to ~1 as the concentration of FL increased from 0.25 to

Table 1. *Steady-state tubular fluid-to-bath ratio and transepithelial secretion rate of FL at different FL concentrations*

FL Concentration, μM	TF/B	Secretion Rate of FL, fmol·min^{-1}·mm^{-1}
0.25	4.9 ± 1.0	17.6 ± 3.6
0.5	3.9 ± 0.7	27.6 ± 4.7
1.0	3.4 ± 0.6	47.4 ± 8.0
2.0	2.9 ± 0.5	80.6 ± 13.1
5.0	2.5 ± 0.6	171.3 ± 38.7
10.0	1.3 ± 0.3	183.3 ± 36.0

Values are means ± SE (n=10). Each experimental point was obtained from a 60-s average of steady-state secretion of fluorescein (FL) in the experiment. TF/B, tubular fluid-to-bath ratio.

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10 μM, suggesting that the transepithelial secretion involved a carrier-mediated process in which carriers were becoming saturated at high concentrations of substrate. Indeed, four of the tubules showed TF/B values of less than 1.0 with 10 μM FL in the bath, indicating that the secretory process was already saturated in these tubules. The kinetic profile of transepithelial secretion of FL by the S2 segment of proximal tubules can be adequately described by the following equation

$$J_{FL} = \frac{J_{max}[FL]_{bath}}{K_t + [FL]_{bath}} \qquad (1)$$

This equation has the same form as the Michaelis-Menten relationship, where J_{FL} is net transepithelial secretion rate at steady state, J_{max} is the maximal transepithelial secretion rate, and K_t is the FL concentration at one-half J_{max}. Kinetic analysis revealed a K_t of ~4 μM and a J_{max} of ~280 $fmol \cdot min^{-1} \cdot mm^{-1}$ (Fig. 3*B*). Subsequently, we used FL as the OA substrate at a concentration of 1 μM, which is well below the K_t, throughout the rest of our studies.

Inhibition of transepithelial secretion of FL by PAH. In recent years, FL has been used as a model substrate for the peritubular OA/DC exchanger on the assumption that the basolateral uptake of FL utilizes the classic OA (PAH) transporter. Increasing evidence supports the idea that basolateral uptake of FL by proximal tubules involves and is limited to the PAH transporter (19, 23, 24). However, we wished to establish that transepithelial secretion of FL involves the same transport system as that for PAH. That this is the case is indicated by the data shown in Fig. 4. The transport rate of FL was decreased when PAH was added during the steady-state secretion of 1 μM FL. The inhibition increased with increasing PAH concentration and was completely reversed after removal of PAH. The kinetic profile suggested competitive inhibition by PAH. The kinetic parameters were calculated by a modification of the isotope dilution procedure of Malo and Berteloot (7) according to the following equation

$$J = \frac{J_{max}[^*T]}{K_t\left(\frac{1 + [I]}{K_i + [^*T]}\right)} + D[^*T] \qquad (2)$$

where J, J_{max}, and K_t are as previously defined, [I] is the concentration of inhibitor (PAH), K_i is the Michaelis constant of the inhibitor, [*T] is the concentration of FL, and D is a coefficient describing nonsaturable transport (passive diffusion). *Equation 2* can be rearranged to give the following

$$J = \frac{J_{app}[^*T]}{K_{i\,app} + [I]} + C \qquad (3)$$

where J_{app} is defined as $(K_i/K_t)J_{max}$, $K_{i\,app}$ is an apparent K_i that is defined as $K_i(1 + [^*T]/K_t)$, and C is a constant derived from D and [*T] reflecting passive transepithelial flux through or between tubule cells. Therefore, knowledge of J, [I], and [*T] permits the calculation of J_{app}, $K_{i\,app}$, and C using nonlinear regression analysis. When [*T] is $<< K_t$, $K_{i\,app} \approx K_i$. Analysis of 12 separate experiments yielded a $K_{i\,app}$ value for the inhibition of FL secretion by PAH of ~108 μM (Fig. 4*B*). This value is almost identical to the concentration for half-

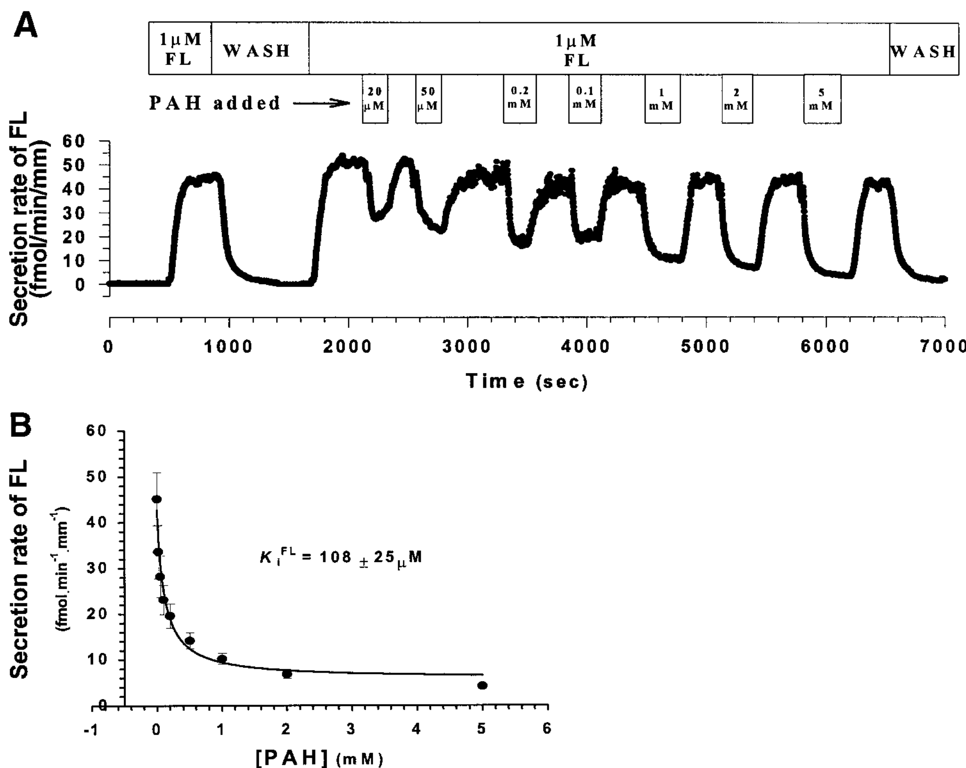

Fig. 4. *A*: a sample tracing of FL transepithelial secretion rate profile in $fmol \cdot min^{-1} \cdot mm^{-1}$ from an isolated perfused S2 segment of rabbit proximal tubules in response to addition of various concentrations of *p*-aminohippurate (PAH) to bath. FL concentration was 1 μM. Addition of PAH to bath during steady secretion of FL resulted in a concentration-dependent reversible inhibition profile. *B*: effect of increasing PAH concentration (shown in mM on abscissa) on steady-state transepithelial secretion rate of 1 μM FL by perfused S2 segments of rabbit renal proximal tubules (shown in $fmol \cdot min^{-1} \cdot mm^{-1}$ on ordinate). Each point represents mean ± SE (n =12, except at PAH concentrations of 0.02 and 0.05 mM, where n = 11). Kinetic parameters, $K_{i\,app}$ and J_{app}, were derived for each tubule with a nonlinear regression algorithm (Enzfitter, Biosoft). Line fitted to data was calculated by a modification of isotope dilution procedure of Malo and Berteloot (7) using averaged kinetic parameters obtained from each tubule.

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maximum transport (K_t) concentration of PAH of ~110 μM reported previously for S2 segments of rabbit proximal tubules (6, 17). The similarity between these kinetic parameters strongly supports the idea that FL and PAH share the same transport system. In addition, transepithelial secretion of 1 μM FL decreased from 45 ± 6 to 4 ± 1 $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$ (91% inhibition) (Fig. 4*B*), when 5 mM PAH was added to the bathing medium. This finding further indicates that transepithelial secretion of FL is essentially limited to the PAH transport system. In other words, it indicates that less than 10% of transepithelial secretion of FL is by some other pathways (e.g., passive paracellular flux).

Influence of basolateral Na-DC cotransporter on transepithelial secretion of FL in the absence of exogenous αKG. The basolateral Na-DC cotransporter plays an important role in the tertiary transport model for peritubular uptake of OA by helping to maintain the in > out gradient for αKG that otherwise could be dissipated during exchange for peritubular OAs (9, 13, 16). However, the actual extent to which recycling of αKG by the basolateral Na-DC cotransporter contributes to the transepithelial secretion of OAs is not certain. To obtain a quantitative assessment of the importance of reuptake by the basolateral Na-DC cotransporter of αKG that has been exchanged for FL, we inhibited this cotransporter with LiCl in the absence of exogenous αKG. We added 2 mM LiCl to the bathing medium in the absence of exogenous αKG while measuring the transepithelial secretion of 1 μM FL. This concentration of LiCl has been shown to inhibit the Na-DC cotransporter (26). When LiCl was added to the bathing medium, FL secretion decreased from the control value of ~54 to 41 $\text{fmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1}$, a decrease of ~23% (Fig. 5). This inhibition was reversible upon removal of LiCl from the bathing medium. In contrast, when the same concentration of LiCl was added to the luminal perfusion solution, it had no effect on FL secretion (Fig. 5). Taken together, these results indicate that the inhibitory effect of LiCl when added to the bath is likely limited to inhibition of αKG recycling by the basolateral Na-DC cotransporter rather than to other metabolic effects of any LiCl that might have entered the cells via the Na-DC cotransporters located on either basolateral or luminal side. Therefore, we conclude that recycling of αKG by the basolateral Na-DC cotransporter contributes ~25% to net OA secretion.

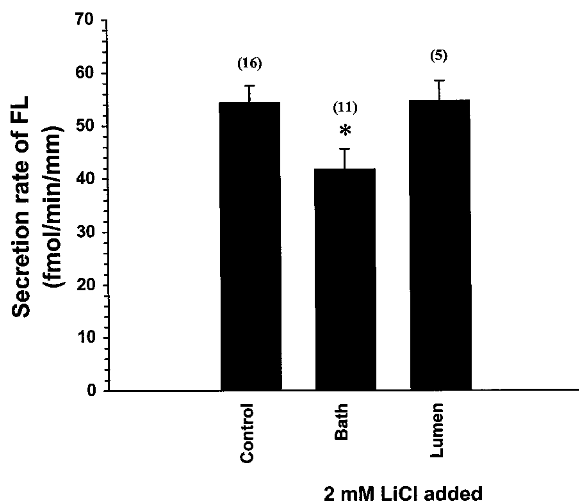

Fig. 5. Effect of 2 mM LiCl added to bath or perfusion solution (lumen) on steady-state transepithelial secretion of 1 μM FL by isolated perfused S2 segments of rabbit renal proximal tubules. Values are means ± SE. Numbers in parentheses indicate numbers of experiments. * Significantly different from control group ($P < 0.05$) as determined by ANOVA.

Influence of exogenous basolateral αKG on transepithelial secretion of FL. Most previous studies on mammalian proximal tubules used nonphysiologically high concentrations (100 μM) of dicarboxylates, αKG or glutarate, for preloading the cells to maximize the stimulatory effect on OA transport in a dicarboxylate-free medium (2, 20). This protocol does not take into consideration the effect of the physiologically available αKG in the plasma, ~10 μM (14), on OA transport. Although Pritchard (11) showed that the addition of 10 μM αKG to the medium bathing rat renal cortical slices caused a 30% increase in PAH uptake, the experiments were performed under nonphysiological conditions (i.e., at room temperature, and in nutrient-free and bicarbonate-free phosphate buffer), conditions that are likely to compromise cellular metabolism and rates of OA transport. Therefore, we sought to evaluate the extent to which exogenous αKG influences FL transport under conditions resembling as closely as possible those to which proximal tubules are exposed in vivo (i.e., nutrient-rich bicarbonate buffer at 37°C).

We first determined the influence of exogenous peritubular αKG on steady-state transepithelial secretion of 1 μM FL by adding increasing concentrations of αKG to the bathing medium. Figure 6 is a typical profile of transepithelial secretion of 1 μM FL by an isolated perfused S2 segment of proximal tubules in response to exogenous peritubular αKG at concentrations ranging from 5 μM to 1 mM. As shown in Fig. 6 and summarized in Table 2, the addition of αKG to the bathing medium affected FL secretion in a biphasic manner. Concentrations of αKG below 200 μM significantly stimulated FL secretion; concentrations of 500 μM and above significantly inhibited it in a concentration-dependent manner. These findings support the idea that high concentrations of dicarboxylates interact competitively with OAs at the extracellular face of the OA/DC exchanger. Maximum stimulation of secretion (~40–50%) was found at αKG concentrations ranging from 10 to 100 μM. Upon removal of 1 mM αKG from the bathing medium, there was an abrupt increase in FL secretion above the control level, presumably due to the accumulation of αKG within the cells following the incubation at high concentrations of αKG (i.e., the equivalent of preloading renal tubules with a high concentration of αKG). This increase was transient and gradually decreased to the control level in the absence of exogenous αKG (Fig. 6). The finding that 10 μM of peritubular αKG stimulated net secretion of FL ~40% (Table 2) indicates that under physiological conditions uptake of exogenous αKG by the basolateral Na-DC cotransporter can support ~29% of net OA secretion; i.e.,

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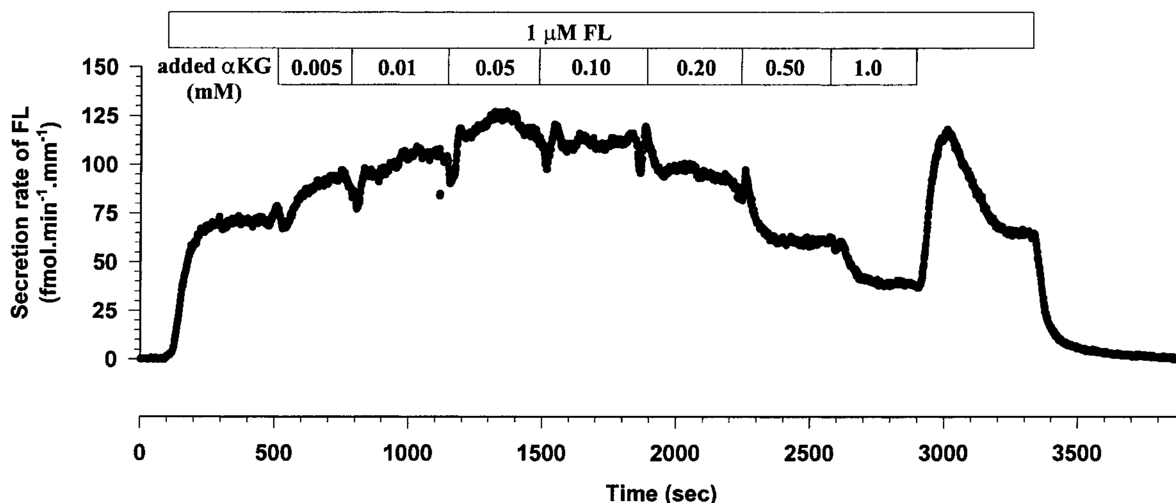

Fig. 6. A representative tracing showing a biphasic effect of α-ketoglutarate (αKG) in bath on transepithelial secretion rate of FL in fmol·min^{-1}·mm^{-1} by an isolated perfused S2 segment of rabbit proximal tubules. FL concentration was 1 μM. Upon removal of 1 mM αKG from the bathing medium, there was an abrupt increase in FL secretion above the control level, presumably due to the accumulation of αKG following the incubation at a high concentration of αKG; i.e., effect of preloading renal tubules with a high concentration of αKG. This increase was transient and gradually decreased to the control level in absence of exogenous αKG.

[(increase in secretion in presence of 10 μM αKG in bath from the "basal" state)/(net secretion in presence of 10 μM αKG in bath)] × 100%. Although it appeared most likely that αKG that entered the tubule cells via the basolateral Na-DC cotransporter stimulated transepithelial FL secretion by countertransport at the basolateral membrane, it was also possible that stimulation could have resulted from metabolism of αKG. To be certain that this was not the case, in another set of experiments, we examined the effects of glutarate or αKG in the bath on FL secretion by the same tubule. Glutarate is not significantly metabolized by the renal cells (10) and is one of the few dicarboxylates other than αKG that is exchanged for PAH at the basolateral membrane (9). As shown in Fig. 7, the addition of 10 μM glutarate to the bath stimulated transepithelial secretion of FL to an extent similar to that of 10 μM αKG (∼37% and ∼24% of control, respectively). There was no significant difference between the stimulation produced by glutarate and αKG. In addition, 1.0 mM glutarate, like 1.0 mM αKG, inhibited the secretion (∼57% and ∼51% of control, respectively). Therefore, αKG that enters the renal tubule cells via the basolateral Na-DC cotransporter apparently stimulates net FL secretion by exchange for FL at the basolateral membrane.

Influence of exogenous luminal αKG on transepithelial secretion of FL. In rabbit renal tissue, the luminal Na-DC cotransporter, which has been cloned and se-

Table 2. *Effect of basolateral αKG on steady-state transepithelial secretion of 1 μM FL*

αKG, mM	Secretion Rate of FL, fmol·min^{-1}·mm^{-1}	% Control
Control	54.4 ± 3.1 (15)	100
0.005	67.2 ± 4.1* (14)	123
0.01	75.9 ± 5.1* (15)	139
0.05	81.8 ± 6.0* (14)	150
0.1	73.3 ± 7.0* (15)	135
0.2	57.5 ± 5.7 (15)	106
0.5	37.1 ± 3.9* (15)	68
1.0	23.8 ± 2.7* (14)	44
Preload	79.8 ± 7.3* (13)	147

Values are mean ± SE. Each experimental point was obtained from a 60-s average of steady-state transepithelial secretion of FL in the experiment. Numbers in parentheses indicate number of experiments. For preload data, each experimental point was a 10-s average of the peak secretion after 1 mM α-ketoglutarate (αKG) was removed from the bath as shown in Fig. 6. * Significantly different from control ($P < 0.05$) as determined by ANOVA.

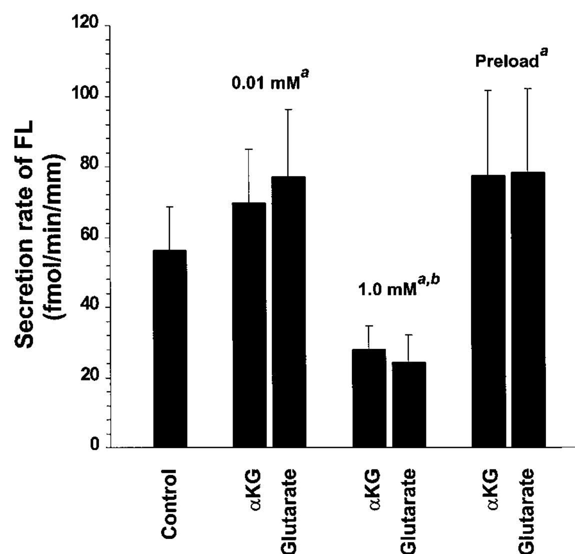

Fig. 7. Effect of αKG or glutarate added to bath on transepithelial secretion of 1 μM FL by isolated perfused S2 segments of rabbit proximal tubules. Concentrations of αKG or glutarate are indicated above each pair of bars. Preload group represents the result obtained after removal of 1 mM αKG or glutarate from the bath as described in Fig. 6. Values are means ± SE (n = 7). No difference was observed between effects of αKG and glutarate. [a]Significantly different from control group ($P < 0.05$). [b]Significantly different from other experimental groups ($P < 0.05$). Differences were determined by ANOVA.

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quenced (8), shows a higher capacity than the basolateral Na-DC cotransport. Because αKG is also filtered, it can be readily reabsorbed from the lumen by the luminal Na-DC cotransporter. Therefore, we hypothesized that the luminal uptake of filtered αKG could be as important as, or perhaps more important than, the uptake of αKG across the basolateral membrane in establishing the in > out αKG gradient for basolateral OA/DC exchange. To evaluate this possibility, we explored the effects of αKG in the lumen on net FL secretion by isolated, perfused S2 segments of proximal tubules in the absence of αKG in the bathing medium. As shown in Fig. 8, addition of αKG to the perfusion solution stimulated steady-state transepithelial secretion of 1 μM FL at every concentration tested. However, at the presumed physiological luminal αKG concentrations of ∼25 to 50 μM, FL secretion was stimulated by only ∼15–20%. From these results, we suggest that luminal uptake of αKG via the luminal Na-DC cotransporter can contribute to transepithelial secretion of OAs by ∼15%; i.e., [(the increase in secretion in the presence of 50 μM αKG in the lumen from the "basal" state)/(net secretion in the presence of 50 μM αKG in the lumen)] × 100%. The data also indicate that at physiological exogenous αKG concentrations, the basolateral Na-DC cotransporter is more effective than the luminal Na-DC cotransporter in promoting OA secretion.

Influence of both basolateral and luminal exogenous αKG on transepithelial secretion of FL. Under normal in vivo conditions, the renal tubule cells are always exposed to dicarboxylates on both basolateral and luminal sides. Transepithelial secretion of OAs, therefore, occurs under conditions where exchangeable dicarboxylates are distributed at steady state within the cells by metabolism and continuous uptake from both sides of the tubule cells. To evaluate the contribution of both basolateral and luminal Na-DC cotransporters to transepithelial secretion of OAs, we examined the

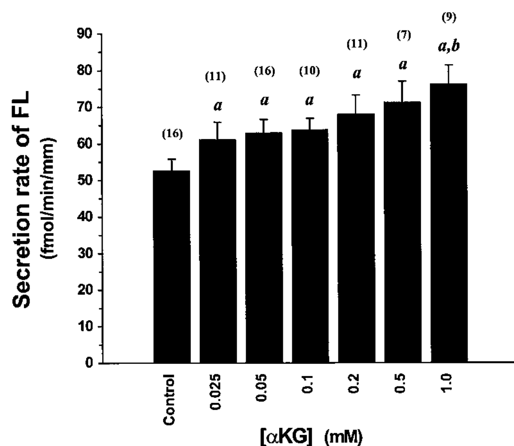

Fig. 8. Effect of αKG added to perfusion solution on transepithelial secretion of 1 μM FL by isolated perfused S2 segments of rabbit proximal tubules. Values are means ± SE. Numbers in parentheses indicate numbers of experiments. [a]Significantly different from control group ($P < 0.05$). [b]Significantly different from groups at a concentration of 0.2 mM and below ($P < 0.05$). Differences were determined by one-way, paired t-test.

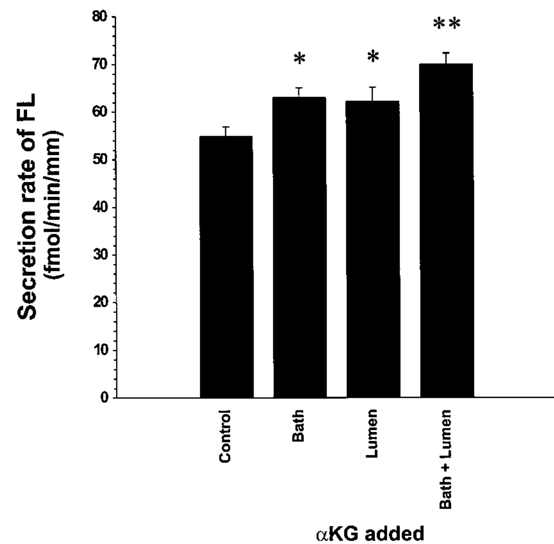

Fig. 9. Effect of αKG added to bath, perfusion solution (lumen), or both on the transepithelial secretion of 1 μM FL by isolated perfused S2 segments of rabbit renal proximal tubules. Values are means ± SE ($n = 9$). *Significantly different from control group ($P < 0.05$). **Significantly different from other experimental groups in which αKG was added either to bath or perfusion solution ($P < 0.05$). Differences were determined by ANOVA.

effects of αKG in the lumen, in the bath, and in both lumen and bath simultaneously on steady-state transepithelial secretion of 1 μM FL by the same individual S2 segment of proximal tubules. The concentrations of αKG added were 10 μM to the bath and 50 μM to the perfusion solution, on the assumption that the latter concentration could possibly be in tubular fluid reaching the S2 segment in vivo. The results are shown in Fig. 9. Addition of αKG to either the luminal or the basolateral side produced an increase in transepithelial secretion of 1 μM FL by ∼13 or 15%, respectively. Interestingly, when the tubule was exposed on both sides to these same concentrations of αKG, the secretion increased to 28%. These data indicate that αKG that enters the cells via the luminal Na-DC cotransporter can further support transepithelial secretion of OAs by increasing the cellular pool of exchangeable αKG at the basolateral membrane.

DISCUSSION

In the present study, we evaluated the transepithelial secretion of the organic anion FL by isolated, perfused S2 segments of rabbit renal proximal tubules in real time by using a specially constructed epifluorescence system in which the collecting pipette functioned as a flow-through cuvette. The studies were also performed under conditions that were as close to physiological as possible, i.e., nutrient-enriched, bicarbonate-buffered bathing and perfusing solutions at 37°C. Initially, we demonstrated that the steady-state transepithelial secretion of FL saturated with an apparent K_t of ∼4 μM and J_{max} of ∼280 fmol·min^{-1}·mm^{-1}. These values were similar to those reported by Sullivan et al. (19) for the basolateral uptake of FL by nonperfused S2 segments of rabbit renal proximal tubules

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(K_t = 10 μM; J_{max} = ~ 498 fmol·min^{-1}·mm^{-1}). Although these authors had doubts about the reliability of the value for J_{max} (19), the similarity between the values for perfused tubules in the current study and those for nonperfused tubules in the earlier study (19) strongly suggests that basolateral transport into the cells is the rate-limiting step for transepithelial transport. Our initial studies also confirmed the assumption that FL transport occurs by the classic OA (PAH) pathway, for PAH inhibited the transepithelial FL transport with an apparent K_i almost identical to the K_t reported previously for PAH transport at the basolateral membrane of this same rabbit tubule segment (6, 17). In the present study, we used the parallel measurements of fluorescence from TMRD in the lumen only to check for leaks during the perfusion. However, with higher concentrations of TMRD in the perfusate, it would be possible to make online measurements of volume change resulting from tubular reabsorption of perfusate.

The use of epifluorescence microscopy to study transepithelial secretion of FL in perfused tubules in real time also allowed us to investigate directly the extent to which the activity of the Na-DC cotransporters located on both the basolateral and luminal membranes contributed to the net transepithelial secretion of OAs. The tertiary active transport model for OA transport at the basolateral membrane suggests that αKG is recycled through the parallel activity of the OA/DC exchanger and the basolateral Na-DC cotransporter (12). The recent study by Welborn et al. (24) on nonperfused rabbit S2 renal proximal tubules indicates that reuptake by the basolateral Na-DC cotransporter of αKG that has moved out of the cells in exchange for FL accounts for ~25% of the initial rate of basolateral FL uptake in the absence of exogenous αKG. Similarly, in the current study on perfused tubules, inhibition of the basolateral Na-DC cotransporter indicated that reuptake of αKG by this transporter accounts for ~25% of the steady-state transepithelial secretion of FL in the absence of exogenous αKG. Therefore, the parallel activities of the basolateral OA/DC exchanger and the basolateral Na-DC cotransporter recycling αKG apparently account for the maintenance of ~25% of the outwardly directed gradient for αKG and the corresponding basolateral uptake and transepithelial secretion of OA in the absence of exogenous αKG.

Under physiological conditions in vivo, renal tubules are exposed to ~10 μM αKG from the blood (bathing medium) side. In previous work on nonperfused rabbit S2 renal proximal tubules, the addition of this concentration of αKG to the bathing medium (which was identical to that used in the present study) containing 1 μM FL led to an increase of ~75% in the initial rate of FL uptake (24). In the present study with perfused tubules, the addition of 10 μM αKG to the bathing medium containing 1 μM FL led to an increase of ~15% to ~40% in the steady-state transepithelial secretion of FL (Table 2; Figs. 7 and 9). The difference in degree of stimulation between our current study on perfused tubules and the previous one on nonperfused tubules (24) may reflect differences in: *1*) transport measured (steady-state transepithelial secretion vs. initial rate of basolateral uptake); *2*) intracellular distribution of αKG taken up from the bath because of differences in the metabolic state of perfused vs. nonperfused tubules; and *3*) the exchangeable intracellular pool of αKG in tubules from different rabbits. The variability between tubules from different rabbits was particularly marked in the current study with perfused tubules (compare data in Table 2 and Figs. 7 and 9). These differences may indeed reflect differences in metabolic state and the available exchangeable intracellular pool of αKG produced by metabolism. This possibility is lent some credence by the observation that glutarate, which is not significantly metabolized (10), tended to produce a slightly higher stimulation of FL transport at 10 μM and inhibition of FL transport at 1 mM than αKG at the same concentrations (Fig. 7).

In the present study on perfused S2 segments of rabbit proximal tubules, it was possible to evaluate the contribution of αKG transport into the cells by the luminal Na-DC cotransporter to the transepithelial secretion of FL in real time. We found that the addition of a concentration of αKG to the lumen that might be expected to be present physiologically (assuming that normal fluid absorption concentrates the filtered αKG before it reaches the S2 segment) produced a significant increase in steady-state net secretion of FL in the absence of αKG in the bathing medium. This stimulation was demonstrated in the presence of bicarbonate-buffered perfusing and bathing solutions, which are essential to maintaining physiological levels of tricarboxylic acid cycle intermediates, such as αKG, in the cells and, thus, normal levels of OA transport (18). In a previous study on perfused S2 segments, Dantzler and Evans (5) also showed that the addition of αKG or glutarate to the lumen could stimulate net transepithelial secretion of radiolabeled PAH in the absence of αKG in the bathing medium. This stimulation was prevented if the luminal Na-DC cotransporter was inhibited by the simultaneous inclusion of LiCl in the perfusate with the αKG or glutarate. However, in this previous study, the stimulation was apparent only with very high concentrations of αKG or glutarate in the lumen and only when the control rate of net steady-state PAH secretion was depressed by using bicarbonate-free perfusate (5). In retrospect, it appears that relatively high concentrations of malate and citrate in the bicarbonate-buffered solutions may have prevented αKG uptake from the lumen by competing for the luminal Na-DC cotransporter. Indeed, we found during the present study that when the bathing solution contained malate and citrate in the presence of a 10 μM concentration of αKG, the usual stimulatory effect on FL secretion was reduced, presumably by competition between malate, citrate, and αKG for the basolateral Na-DC cotransporter (unpublished observations). Moreover, the simple removal of malate and citrate from the bathing medium produced an increase in FL secretion (unpublished observations). In this case, malate and

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citrate were probably inhibiting reuptake of αKG by the basolateral Na-DC cotransporter.

Although kinetic data are not available for the transport of αKG itself by either the luminal or basolateral Na-DC cotransporter, studies of succinate transport by brush-border membrane vesicles (BBMV) and BLMV provide some information on the possible affinities and capacities of these two transporters for αKG (27). In BBMV, the K_t and J_{max} for succinate are ~600 μM and ~90 $nmol \cdot min^{-1} \cdot mm^{-1}$, respectively; in BLMV, they are ~10 μM and ~5 $nmol \cdot min^{-1} \cdot mm^{-1}$ (16). The inhibitory effects of αKG on succinate transport suggest that the K_t values for it may be similar to those for succinate (27). These data suggest that physiological concentrations of αKG in the lumen should certainly be taken up by the luminal Na-DC cotransporter to contribute to the gradient for FL/αKG exchange at the basolateral membrane, as clearly occurred in the present study. However, in general, 50 μM αKG in the lumen had less stimulatory effect on transepithelial FL secretion (in the absence of αKG in the bath) than did 10 μM αKG in the bath (in the absence of αKG in the lumen). This observation might be related to the anatomic proximity of the basolateral Na-DC cotransporter to the basolateral OA/DC exchanger. The αKG transported into the cells via the basolateral Na-DC cotransporter might be supplied relatively directly to the OA/DC exchanger, whereas the αKG transported into the cells by the luminal Na-DC cotransporter might contribute to both the intracellular pool of exchangeable αKG for the OA/DC exchanger and to other cellular metabolic events.

In the previous study on the role of the luminal uptake of αKG in the process of PAH secretion, the time course of the radioisotopic measurements markedly limited the sensitivity of the determinations of PAH secretion, and it was not feasible to study the effects of αKG in the lumen and bathing medium simultaneously (5). In the present study, it was possible to determine the effect of αKG in the lumen alone, the bath alone, and in both the lumen and bath simultaneously on steady-state transepithelial FL secretion in the same individual tubules. The data revealed a significant additive effect on FL secretion when αKG was in both the lumen and the bath at approximately physiological concentrations (Fig. 9). Therefore, the luminal uptake of αKG can play a role in maintaining secretion of OA under physiological conditions with αKG present at the basolateral side. However, it should be noted that in the series of experiments reported in Fig. 9, the degree of stimulation of FL secretion by 10 μM αKG in the bathing medium was substantially less than that in other experiments (for example, Table 2). Therefore, although uptake of αKG from the lumen can contribute to OA secretion even with αKG in the bath, the contribution may be less when the basolateral uptake is more effective.

The present observations on the effects of αKG permit us to estimate the extent to which the basolateral and luminal Na-DC cotransporters may contribute to net transepithelial secretion of OAs under normal physiological conditions. Recycling of αKG by the basolateral Na-DC cotransporter supports ~25% of the "basal" secretion in the absence of exogenous αKG. The basolateral Na-DC cotransporter can also support a further ~30% (average value from all experiments) of OA secretion by uptake of exogenous αKG from the physiological basolateral concentration of 10 μM. Therefore, the activity of the basolateral Na-DC cotransporter is responsible for supporting ~42% of the transepithelial secretion of OAs; i.e., {[(increase in secretion in presence of 10 μM αKG in bath from the "basal" state) + (difference in secretion in presence of 2 mM LiCl in bath from the "basal" secretion)]/(net secretion in presence of 10 μM αKG in bath)} × 100%. The luminal Na-DC cotransporter can support an additional ~20% (average value from all experiments) of OA secretion by uptake of αKG from the luminal fluid, if the luminal concentration is 50 μM. As noted above, the effect of αKG uptake by both the luminal and basolateral Na-DC cotransporters on OA secretion can be additive with physiological concentrations of αKG in lumen and bath. If this is the case and if we use the average individual values obtained, we can conclude that the luminal and basolateral Na-DC cotransporters together directly support ~50% of the net transepithelial OA secretion; i.e., {[(increase in secretion in presence of 10 μM αKG in bath from the "basal" state) + (difference in secretion in presence of 2 mM LiCl in bath from the "basal" secretion) + (increase in secretion in presence of 50 μM αKG in lumen from the "basal" state)]/(net secretion in presence of 10 μM αKG in bath and 50 μM αKG in lumen)} × 100%. We assume that this occurs by these transporters establishing and maintaining ~50% of the outwardly directed gradient for αKG that is responsible for driving the OA/DC exchange at the basolateral membrane. The remaining ~50% would be maintained by metabolic production of αKG in the cells.

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