Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy

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Masereeuw, Rosalinde, Frans G. M. Russel, and David S. Miller. Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. Am. J. Physiol. 271 (Renal Fluid Electrolyte Physiol. 40): F1173–F1182, 1996.—Previous studies with p-aminohippurate (PAH) and fluorescein (FL) have shown that cellular uptake and tubular secretion of organic anions is driven by indirect coupling to sodium. Here we used killifish proximal tubules and laser-scanning confocal microscopy to study the transport of a larger organic anion, fluorescein-methotrexate (FL-MTX, mol mass 923 Da). When tubules were incubated in medium containing 2 μM FL-MTX, dye accumulated in both cells and tubular lumens. At steady state, luminal fluorescence was 4–5 times higher than cellular fluorescence. Ouabain (0.1 mM) did not affect cellular or luminal fluorescence, and replacement of medium sodium by N-methylglucamine had only a modest effect; preincubation with glutarate had no effect. KCN did not affect cellular uptake but abolished secretion into the lumen. Uptake and secretion of FL-MTX were inhibited by micromolar concentrations of other organic anions (MTX, folate, probenecid, bromocresol green, bromosulfophthalein), but 1 mM PAH had a relatively small effect. FL-MTX secretion into the lumen was inhibited by leukotriene C₄, cyclosporine A, and verapamil, none of which affected FL transport. Thus a substantial component of FL-MTX secretion is Na independent and ouabain insensitive. Both the basolateral and luminal steps in the Na-independent pathway differ from those usually associated with FL and PAH secretion.

THE CLASSIC ORGANIC ANION transport system in vertebrate renal proximal tubule is responsible for the excretion of a large number of metabolic wastes and xenobiotics (reviewed in Ref. 25). Previous studies of organic anion transport in a number of preparations have demonstrated that secretion is sodium dependent and ouabain sensitive. Membrane vesicle studies and experiments with intact renal tissue have disclosed the mechanistic basis for these observations: indirect coupling of uptake at the basolateral membrane to Na through Na-divalent organic anion cotransport and organic anion exchange (25). These studies utilized small organic anions, such as the model compounds p-aminohippurate (PAH) and fluorescein (FL), as substrates. However, the proximal tubule handles a wide range of anionic compounds, some quite large. It is generally assumed that these larger compounds are handled by the same mechanisms as smaller organic anions, since PAH and probenecid can reduce their transport and since many large organic anions are themselves potent inhibitors of FL and PAH transport (25).

In the present study, we used confocal fluorescence microscopy to examine the transport of three fluorescent organic anions by killifish renal proximal tubules (Fig. 1). One anion, FL (mol mass 332 Da), has been studied previously using conventional fluorescence microscopy and has been shown to be secreted by the same Na-dependent mechanism as PAH (18, 19, 27, 28). Both the others, fluorescein-methotrexate (FL-MTX) and sulforhodamine 101 (Texas red free acid), are substantially larger, with molecular masses of 923 and 606 Da, respectively. Although neither has been used as a substrate for renal transport studies, several reports indicate that the parent compound of one, MTX, undergoes net secretion in mammalian proximal tubule and that MTX uptake and secretion are sensitive to inhibition by probenecid and PAH (1, 3, 4, 9, 10).

As discussed previously, renal tissue from teleost fish offers several advantages for the study of secretory transport mechanisms (15, 24). Teleost kidneys contain a high proportion of proximal tubules that are easily isolated and that remain viable for long periods. When tubules are isolated, broken ends rapidly reseal to form a closed, fluid-filled luminal compartment that only communicates with the medium through the tubular epithelium. Thus this tissue has the appropriate geometry for the study of transepithelial secretion in intact tubules. Moreover, secretory transport mechanisms found in teleost tubules appear to be identical to those found in mammalian proximal tubules (18, 16, 26). Finally, when teleost tubules are used along with fluorescent substrates and quantitative fluorescence microscopy, the mechanisms driving both uptake by the cells and secretion into the tubular lumen can be examined (17, 18, 26).

The present data show that killifish renal proximal tubules secrete FL-MTX and sulforhodamine 101 by a two-step process. However, unlike FL, a substantial fraction of the secretion of FL-MTX and sulforhodamine 101 is driven by Na-independent mechanisms.

MATERIALS AND METHODS

Chemicals. FL and FL-MTX were purchased from Molecular Probes (Eugene, OR). Bromocresol green (BCG), bromosulfophthalein (BSP), daunomycin, leukotriene C₄ (LTC₄), probenecid, tetraethylammonium (TEA) chloride, sulforhodamine 101 free acid, and verapamil were purchased from Sigma Chemical (St. Louis, MO). Cyclosporin A (CSA) was obtained from Dr. G. Fricker (Sandoz Pharma, Basel, Switzerland). All

F1173
other chemicals were obtained from commercial sources at the highest purity available.

Animals and tubule preparation. Killifish (Fundulus heteroclitus) were collected near Duke University Marine Laboratory (Beaufort, NC) and maintained in tanks with recirculating, artificial sea water (18°C) at the National Institute of Environmental Health Sciences.

Renal tubular masses were isolated in a marine teleost saline based on that of Forster and Taggart (8), containing (in mM) 140 NaCl, 2.5 KCl, 1.5 CaCl₂, 1.0 MgCl₂, and 20 tris(hydroxymethyl)aminomethane at pH 8.0. In the Na-depletion experiments, Na was replaced with ¿V-methylglucamine. All experiments were carried out at 18–20°C. Under a dissecting microscope, each mass was teased with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free from adjacent cells with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free from adjacent cells with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free from adjacent cells with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free from adjacent cells with fine forceps.

Confocal fluorescence microscopy. Tubules in the chamber were mounted on the stage of a Zeiss model 410 inverted laser-scanning confocal microscope and viewed through a Zeiss ×40 plan-neofluor water immersion objective (NA = 1.3). The microscope was fitted with an Ar-Kr laser providing light at 488 and 568 nm. For measurements of FL and FL-MTX fluorescence, the 488-nm laser line, a 510-nm dichroic filter, and a 515-nm long-pass emission filter were employed. For measurements of sulforhodamine 101 fluorescence, the 568-nm laser line, a 575-nm dichroic filter, and a 590-nm long-pass emission filter were employed. Most images were collected with a zoom setting of 2 (0.313 μm/pixel). Neutral density filters passing 1 or 3% of the light and 20% laser power were used to minimize photobleaching. Preliminary experiments showed that, under these conditions, fluorescence intensities in cells and tubular lumens were reduced by <5% in consecutive 8-s scans. With these settings and with photomultiplier gain adjusted so that the average pixel intensity in the lumens of control tubules was 150–200 (on a scale of 0–255), tissue autofluorescence was usually undetectable.

To obtain an image, dye-loaded tubules in the chamber were viewed under reduced, transmitted-light illumination, and a single proximal tubule with well-defined lumen and undamaged epithelium was selected. The plane of focus was adjusted to cut through the center of the tubular lumen. Then, in confocal fluorescence mode, a single 8-s scan of the tubule was collected. The confocal image (512 × 512 × 8 bits) was viewed on a high-resolution monitor and saved to optical disk. Fluorescence intensities were measured from stored images using an Apple Power Macintosh 7100 computer and NIH Image version 1.58 software, as described previously (16, 17). Briefly, three to five adjacent cellular and luminal areas (at least 200–400 pixels each) were selected from each tubule. After background subtraction, the average pixel intensity for each area was calculated. Then the lumen-to-cell fluorescence ratio for each pair of adjacent areas was calculated. The values used for that tubule were the means of all measured areas.

Metabolism. Available data from mammalian studies suggest that we should expect minimal metabolism of FL-MTX by fish tubules in the present short-term incubation studies carried out at 18°C. In vivo, mammals only slowly metabolize MTX to its 7-OH derivative, which can be eliminated in urine and bile (see discussion in Ref. 6). In addition, in perfused rabbit tubules that were bathed for 1 h in medium with [3H]MTX, metabolites accounted for only a small percentage of label in luminal fluid (1). We incubated killifish tubules (pooled tubules from 4 fish) in media with 2 μM [3H]MTX (activity 1 μCi/ml) or 2 μM FL-MTX and analyzed water extracts using standard thin-layer chromatography procedures. For analysis of [3H]MTX, cellulose-coated plates were developed in 0.1 M phosphate buffer (pH 7.0). Lanes were cut into 1-cm bands, and each band was placed in a vial for extraction and liquid scintillation counting; >95% of the radioactivity migrated with authentic MTX. For analysis of FL-MTX, DEAE-cellulose-coated plates were developed in 0.25 M ammonium bicarbonate/20% acetonitrile (pH 7.0). Plates were illuminated with violet ultraviolet light, and location of green fluorescence was noted. Using this semiquantitative method, we estimate that >90% of the fluorescence on the plate migrated with authentic FL-MTX. No fluorescence could be detected in the region of the plate where FL would have run.

Statistics. Data are given as means ± SE. Means were considered to be statistically different, when P < 0.05 by use of the appropriate paired or unpaired t-test.

RESULTS

In the present experiments, killifish renal proximal tubules were incubated in medium containing FL, FL-MTX, or sulforhodamine 101; confocal fluorescence images were collected, and fluorescence distribution patterns were analyzed to obtain an indication of dye distribution in the tissue. Data are presented as steady-state fluorescence intensity measurements made over the cellular and luminal regions of the tubules, as well as paired lumen-to-cell fluorescence ratios. Two caveats must be kept in mind when interpreting such measurements. First, the signal from a fluorescent probe is sensitive to environment, e.g., pH or solvent polarity. As a result, absolute calibration of dye concentration in a single region of a tissue or a cell is difficult, and the
FL transpport. Previous studies from this laboratory and others have demonstrated that FL, like PAH, can be used as a model substrate for the organic anion transport system in vertebrate renal proximal tubule (18, 19, 27, 28). The data in Fig. 2, obtained by analysis of confocal micrographs of killifish proximal tubules incubated to steady state in medium with 1 μM FL, illustrate several hallmarks of this system. First, secretion is a two-step process. In control tubules, the average fluorescence intensity of the tubular lumen exceeded that of the cells which exceeded that of the medium (Fig. 2). This is the same pattern seen when teleost renal tubules are incubated with colored organic anions, such as chlorophenol red, and dye concentration is estimated by eye or measured by absorption microspectroscopy (12). Thus it is unlikely that the fluorescence distribution pattern seen for FL reports primarily differences in probe environment. Rather, the pattern is due to concentrative transport steps at both the basolateral and luminal membranes. Second, FL transport is highly Na dependent. Replacement of medium Na with N-methylglucamine abolished FL uptake and secretion, and 100 μM ouabain reduced cellular and luminal fluorescence by >85% (Fig. 2). This concentration of ouabain has been previously shown to inhibit Na-K-adenosinetriphosphatase (Na-K-ATPase) in homogenates of teleost renal tubules and to greatly reduce [3H]PAH accumulation in intact tubules (14). Third, the uptake and secretion of FL are sensitive to inhibition by competitor organic anions. As shown in Fig. 2, 1 mM PAH or 0.5 mM probenecid abolished the uptake of 1 μM FL, consistent with transport of the three compounds by a single system.

FL-MTX transport. Confocal micrographs of killifish proximal tubules incubated for 30 min in medium with 2 μM FL-MTX showed fluorescence intensity in the lumen > cells > bath (Fig. 3A). This is the same general pattern of tissue fluorescence seen with other fluorescent and colored organic anions (above). It suggests two uphill steps during secretion: one at the basolateral membrane and the other at the luminal membrane. As has been demonstrated previously for FL and other fluorescent organic anions (17, 19), cellular fluorescence was not uniformly distributed. Fluorescence intensity in the basal region of many cells was noticeably higher than in the apical region, and nuclear fluorescence was below that seen in the rest of the cell (Fig. 3B). This is probably because of accumulation of FL-MTX by mitochondria (14) and other vesicular compartments (19, 20) embedded in cytoplasm and the absence of such compartments in the nucleus. With higher magnification and higher photomultiplier gain settings, punctate sites of FL-MTX fluorescence could be seen throughout the cells (Fig. 3B). In addition, thin confocal sections at high magnification show some areas of relatively high fluorescence associated with the basolateral membrane, suggesting binding of the compound to the plasma membrane (Fig. 3C). In preliminary experiments (data not shown), we followed the time course of FL-MTX accumulation in individual tubules. Cellular fluorescence rose over the first several minutes and then reached a plateau. Luminal fluorescence increased linearly over the first 8 min and reached steady state within 10 min. At steady state, the lumen-to-cell fluorescence ratio averaged 4 to 5. All subsequent measurements of tubule fluorescence were made at steady state, i.e., after 15- to 30-min incubation with fluorescent substrate.

For tubules exposed to 2 μM FL-MTX, replacing medium Na with N-methylglucamine reduced cellular and luminal fluorescence but only by 48 ± 22 and 31 ± 13%, respectively (Fig. 4; significant inhibition, P < 0.05). Although Na depletion had partially reduced both cellular and luminal fluorescence, the lumen-to-cell fluorescence ratio was still well over unity, indicating that secretion continued in the absence of Na. In addition, 100 μM ouabain had no effect on FL-MTX transport (Fig. 4); 10 μM glutarate or 5 mM LiCl plus 10 μM glutarate had no effects (Fig. 4). This concentration of glutarate has been shown to stimulate Na-dependent FL and PAH uptake in teleost proximal tubules (18, 19) and in a variety of mammalian preparations (25); in those experiments, LiCl abolished the glutarate stimulation. FL-MTX secretion was dependent on cellular metabolism, since 1 mM KCN greatly reduced luminal fluorescence (Fig. 4). However, KCN did not significantly reduce cellular fluorescence.

The transport of 2 μM FL-MTX into killifish proximal tubules was also sensitive to inhibition by other organic anions. Although PAH and probenecid reduced FL
MTX uptake and secretion, these organic anions were not nearly as effective as when FL was the substrate. Figure 5 shows that 1 mM PAH reduced both cellular and luminal fluorescence (from FL-MTX) by 40 ± 14 and 27 ± 11%, respectively, and that 0.5 mM probenecid reduced cellular and luminal fluorescence by 71 ± 18 and 35 ± 10% (P < 0.01), respectively. Thus, after exposure to either PAH or probenecid, substantial FL-MTX uptake and secretion were still observed. The parent compound, MTX, caused a concentration-dependent decrease in both cellular and luminal fluorescence (Fig. 5). The concentration of MTX causing 50% reduction of fluorescence in both compartments was ~100 μM. Folate was a less potent inhibitor of FL-MTX transport, with 250 μM inhibiting by slightly <50%. The large organic anions, BSP and BCG, were considerably more potent inhibitors of FL-MTX secretion than MTX or folate (Fig. 5). At 10 μM, BSP and BCG reduced luminal fluorescence by >90%; of the two, only BCG had a significant effect on cellular fluorescence.

In liver, secretion of large amphiphilic organic anions from hepatocyte to bile canaliculus is mediated by one or more multispecific organic anion transporting ATPases (21). These ATPases are distinct from the multidrug resistance (MDR) transporter (p-glycoprotein) that is present at high levels in the canicular membrane of hepatocytes and in the luminal membrane of renal proximal tubule cells (29). The organic anion transporting ATPases are sensitive to inhibition by a variety of anionic compounds. Of these, cysteinyi leukotrienes (LTC) have a particularly high affinity for the transporter, with LTC₄ exhibiting a Michaelis constant of 250 nM (11). Transport on these ATPases is also inhibited by CSA with an inhibitory constant of 3 μM (3). To determine whether a transporter with similar specificity might be driving the transport of large
organic anions from cell to lumen in proximal tubule, we measured the effects of LTC₄ and CSA on FL-MTX transport. Figure 6A shows that LTC₄ at 100 and 300 nM reduced luminal fluorescence by 45 ± 14 and 63 ± 21% (P < 0.01), respectively, but did not significantly affect cellular fluorescence. Based on these results, LTC₄ appears to be the most powerful inhibitor of FL-MTX secretion tested. In addition, when tubules were incubated in Na-free medium and photomultiplier gain was raised to increase sensitivity, 300 nM LTC₄ abolished luminal fluorescence (luminal and cellular fluorescence in 10 tubules incubated in Na-free medium averaged 133 ± 10 and 20 ± 3 fluorescence units, respectively; luminal and cellular fluorescence in 12 tubules incubated in Na-free medium with 300 nM LTC₄ averaged 3 ± 1 and 19 ± 3 fluorescence units, respectively). These data argue that all of the Na-independent transport of FL-MTX from cell to lumen was sensitive to inhibition by LTC₄.

Figure 6A also shows that CSA, at 5 μM, reduced luminal fluorescence by 49 ± 16% but had no significant effects on cellular fluorescence. Surprisingly, FL-MTX transport was also reduced by verapamil, an organic cation that inhibits transport through

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**Fig. 4.** Effects of Na replacement, 0.1 mM ouabain, 10 μM glutarate (GLUT), and 1 mM KCN on the transport of 2 μM FL-MTX. Tubules were incubated for 30 min in medium containing 2 μM FL-MTX and indicated additions; images were collected and analyzed as described in MATERIALS AND METHODS. Data are given as means ± SE for 8–12 tubules from 2 fish. Na-free medium reduced cellular and luminal fluorescence significantly (P < 0.05); KCN reduced luminal fluorescence significantly (P < 0.01).

![Fig. 4](image_url)

**Fig. 5.** Inhibition of FL-MTX transport by organic anions. Tubules were incubated for 30 min in medium containing 2 μM FL-MTX and indicated additions; images were collected and analyzed as described in MATERIALS AND METHODS. Data are given as means ± SE for 6–15 tubules from 1–3 fish. All treatments reduced luminal fluorescence significantly (P < 0.01); PAH, probenecid, and folate reduced cellular fluorescence significantly (P < 0.01); bromocresol green (BCG) reduced cellular fluorescence significantly (P < 0.05); bromosulfophthalein (BSP) did not reduce cellular fluorescence.

![Fig. 5](image_url)
p-glycoprotein (7) but not through the canalicular organic anion ATPase (11). This inhibition was not related to the high affinity that verapamil exhibits for the renal organic cation transport system, since the model organic cation, TEA, at 1 mM, did not reduce FL-MTX transport (Fig. 6A). In contrast, neither LTC₄, CSA, nor verapamil, at the concentrations used, had any effects on the transport of the small organic anion, FL (Fig. 6B). The CSA and verapamil results agree with previously published data for FL transport in killifish tubules (26).

We explored further the effects of verapamil on FL-MTX transport. The data presented above demonstrate that, in contrast to FL, FL-MTX transport was only partially inhibited by 500 μM probenecid. Figure 7 shows that increasing the probenecid concentration above 250 μM caused no further reduction in cellular or luminal fluorescence from FL-MTX. Clearly, at concentrations >250 μM, the probenecid-sensitive component of FL-MTX transport was blocked. Figure 7 also shows that, when tubules incubated in medium with 2 μM FL-MTX were exposed to 500 μM probenecid plus 50
μM verapamil, luminal fluorescence was reduced by 66 ± 24% compared with 500 μM probenecid alone (P < 0.05). Cellular accumulation of FL-MTX was not reduced further by verapamil plus probenecid compared with probenecid alone. Thus verapamil appeared to block a component of FL-MTX transport from cell to lumen that was insensitive to 500 μM probenecid.

Sulforhodamine 101 transport. To determine whether organic anions intermediate in size between FL and FL-MTX are also transported by a Na-independent and ouabain-insensitive system, we followed the transport of sulforhodamine 101 free acid, a fluorescent organic anion with a molecular mass of 606 Da. Figure 8 shows that sulforhodamine 101, like FL and FL-MTX, was secreted into the lumens of killifish tubules. At steady state, the lumen-to-cell fluorescence ratio in control tubules averaged 5 to 8. Ouabain, at 0.1 mM, reduced cellular and luminal fluorescence by 31 ± 9 and 53 ± 12% (P < 0.01), respectively, indicating a ouabain sensitivity part way between that of FL and FL-MTX (Fig. 8). Sulforhodamine 101 transport was also inhibited by 1 mM PAH and 0.5 μM probenecid, with the latter compound being substantially more effective. LTC₄, CSA, and verapamil reduced luminal fluorescence but had no significant effects on cellular fluorescence (Fig. 8). Finally, in ouabain-treated tubules, LTC₄ significantly reduced luminal but not cellular fluorescence (when photomultiplier gain was raised, luminal and cellular fluorescence in 8 tubules incubated in medium with 0.1 mM ouabain averaged 159 ± 19 and 27 ± 4 fluorescence units, respectively; luminal and cellular fluorescence in 9 tubules incubated in medium with 0.1 mM ouabain plus 300 nM LTC₄ averaged 69 ± 11 and 26 ± 3 fluorescence units, respectively). Thus, based on these initial experiments, it appears that transport of sulforhodamine 101 occurs by both ouabain-sensitive and ouabain-insensitive pathways and that, like FL-MTX, a component of sulforhodamine 101 secretion is inhibited by LTC₄, CSA, and verapamil. A substantial portion of the ouabain-insensitive component of sulforhodamine 101 secretion is blocked by LTC₄.

DISCUSSION

Organic anion secretion. The current model for organic anion secretion in renal proximal tubule cells involves uptake indirectly coupled to Na at the basolateral membrane followed by electrical potential-driven facilitated diffusion into the tubular lumen (the “classic” organic anion transport system; Fig. 9). Intracellular processes also affect organic anion transport, since there is evidence for compartmentation of organic anions and a dependence of secretion on an intact microtubular system (19, 20). It follows from this series model of transepithelial transport that maneuvers...
RENAL ORGANIC ANION TRANSPORT

Fig. 9. Mechanisms of organic anion transport in renal proximal tubule. For convenience, mechanisms are shown in two cells; at present, there is no reason to believe that all steps will not be found in every renal proximal tubule cell. Top cell shows transport steps initially established by experiments with renal basolateral and brush-border membrane vesicles (the “classic” Na-dependent, organic anion transport system with fluorescein (FL) as substrate; Ref. 25). These include 1) Na-α-ketoglutarate (α-KG) cotransport, 2) α-ketoglutarate organic anion exchange at the basolateral membrane, and 3) facilitated diffusion at the luminal (brush-border) membrane. Bottom cell shows additional 4) basolateral and 5) luminal steps proposed in the present imaging study of fluorescein-methotrexate (FL-MTX) transport; the ATP dependence of step 5 has yet to be demonstrated. Accumulation of both substrates in intracellular compartments is also shown.

affecting uptake into the cell would be expected to similarly alter secretion into the lumen. Examples of this phenomenon are the increases in cellular FL accumulation and transport into the lumen seen with 10–50 μM glutarate and the decreases in both caused by Na depletion or ouabain treatment (Fig. 2 and Refs. 18, 19). It also follows that, in the absence of other active transport mechanisms, organic anion uptake and secretion should be Na dependent, and this is just what has been reported in studies of PAH and FL transport in intact renal tissue from a number of species (25).

The results of the present confocal imaging study with killifish renal proximal tubules argue for involvement of an additional pathway in the secretion of larger organic anions (Fig. 9). Most of the data were obtained with FL-MTX as substrate, but initial experiments with a second large fluorescent organic anion, sulforhodamine 101, indicate that the transport system handles more than just MTX and derivatives. Tubules incubated in medium with either of these fluorescent solutes exhibited roughly the same pattern of tissue fluorescence as seen with the small organic anion, FL. That is, average luminal fluorescence exceeded average cellular fluorescence and cellular fluorescence exceeded medium fluorescence. Nevertheless, four telling differences were observed between the transport of the small organic anion, FL, and the large organic anions, FL-MTX and sulforhodamine 101. First, at least half of the steady-state accumulation of FL-MTX in cells and tubular lumens remained after medium Na was re-placed by N-methylglucamine. Ouabain had no effect on FL-MTX transport and only partially reduced sulforhodamine 101 transport. In contrast, Na replacement and ouabain exposure nearly abolished the uptake and secretion of FL. Second, although FL-MTX transport was sensitive to inhibition by the same variety of organic anions that affect FL and PAH transport, inhibition by 1 mM PAH and 0.5 mM probenecid appeared to be only partial. Again, this is in contrast to data for FL, which show nearly complete inhibition of transport with those same concentrations of PAH and probenecid. Third, the transport of FL-MTX and sulforhodamine 101 was inhibited by CSA, an uncharged cyclic polypeptide derivative, and by verapamil, an organic cation, neither of which had any effects on FL transport. One thing CSA and verapamil do have in common is that they are inhibitors of some xenobiotic transport ATPases (see below). Finally, the most potent inhibitor of FL-MTX secretion tested was LTC4, which was effective at submicromolar concentrations. LTC4 blocked luminal accumulation of FL-MTX and sulforhodamine 101 but had no effects on cellular accumulation. LTC4 inhibited the Na-independent component of FL-MTX transport and the ouabain-insensitive component of sulforhodamine 101 transport. At the concentrations used, LTC4 had no effects on FL transport. Thus, based on Na dependence and inhibitor specificity, a substantial portion of the secretion of FL-MTX and sulforhodamine 101 is mediated by a transport system very different from that which drives FL secretion. In the next two sections, we consider the nature of the basolateral and luminal steps, focusing on the FL-MTX data.

Basolateral uptake. Organic anions, such as PAH, probenecid, MTX, folate, BSP, and BCG, significantly reduced steady-state cellular accumulation of FL-MTX, in some cases by as much as 80–90%. By this criterion, a large fraction of the total cellular accumulation of FL-MTX appears to be specific. From these data, we can construct a preliminary order of inhibitory effectiveness: BCG > MTX > folate = probenecid > PAH. Surprisingly, verapamil, an organic cation, also reduced cellular accumulation by ~40%. The portion of FL-MTX uptake affected by verapamil was also inhibited by probenecid, since verapamil plus probenecid had no greater effect on cellular accumulation than probenecid alone. Furthermore, the effects of verapamil do not appear to be related to FL-MTX transport on the organic cation system or on a drug transporting ATPase, since neither TEA, CSA, nor LTC4 had any effects on cellular accumulation of FL-MTX. Note that verapamil did not inhibit the cellular accumulation or secretion of FL, which is highly dependent on cellular metabolism and ion gradients. This result argues that verapamil did not reduce FL-MTX uptake by nonspecific mechanisms, such as cellular toxicity.

Cellular accumulation of FL-MTX was not reduced when metabolism was abolished with KCN, although luminal accumulation was inhibited completely. These observations, along with the inhibitor studies discussed above, suggest that cellular accumulation of FL-MTX, although specific, is not energy dependent. Rather, the
high cellular accumulation seen in confocal micrographs could be a result of diffusive entry (simple diffusion and facilitated diffusion) combined with extensive binding to cellular elements. At steady state, the binding component appears to predominate. Two additional observations are consistent with this suggestion: first, confocal micrographs show accumulation of FL-MTX associated with the basolateral membrane of killifish renal proximal tubule cells and intracellular structures (Fig. 3), and, second, preliminary experiments show high levels of specific FL-MTX binding to a fraction of endosomes isolated from rat renal cortex (unpublished observations). Together, these observations suggest that, at a minimum, FL-MTX binds to multiple cellular structures.

Cellular accumulation of FL-MTX was partially reduced when medium Na was replaced by N-methylglucamine but was not affected by a concentration of ouabain that abolished PAH and FL accumulation and that inhibited teleost renal Na-K-ATPase (14). Although the effects of Na replacement could be explained by a small component of uptake being mediated by the Na-coupled system, the insensitivity of uptake to KCN suggests that the Na-independent mechanisms of transport and binding processes that lead to the Na-independent accumulation of large organic anions in renal proximal tubule cells.

Secretion into the lumen. Killifish tubules incubated at steady state in medium containing 2 μM FL-MTX exhibited lumen-to-cell fluorescence ratios of 4 to 5, suggesting concentrative transport of that substrate across the luminal membrane. Consistent with metabolism-driven, concentrative transport, luminal fluorescence was reduced substantially when metabolism was inhibited by KCN. Na replacement reduced luminal FL-MTX by ~40%, but cellular accumulation was similarly reduced. This could mean that Na replacement reduced luminal fluorescence in large part through decreased cellular accumulation. Indeed, many of the effects of the organic anions used as inhibitors of FL-MTX transport could be explained on this basis. For example, Fig. 5 shows that, although both cellular and luminal fluorescence fell with increasing MTX concentration, they fell together. Because we cannot differentiate effects on secretion into the lumen from those on uptake into the cell (above), most of the present data on organic anion effects on FL-MTX transport provide little information about the specificity of the luminal step.

There were, however, three compounds, CSA, verapamil, and LTC4, that decreased luminal fluorescence but had no effects on cellular fluorescence or reduced it only slightly. These compounds had no effects on the cellular or luminal accumulation of FL, indicating that a large component of the cell-to-lumen transport of FL-MTX is not shared with FL. LTC4, CSA, and verapamil also reduced sulforhodamine 101 accumulation in the lumen, suggesting that at least a portion of the cell-to-lumen transport of that dye is through the pathway shared with FL-MTX but not with FL. LTC4 inhibited the Na-independent component of FL-MTX transport and the ouabain-insensitive component of sulforhodamine 101 transport.

The lack of an effect of LTC4 and CSA on the steady-state cellular accumulation of FL-MTX and sulforhodamine 101 requires further comment. One might expect cellular fluorescence to increase when transport from cell to lumen was inhibited. However, many processes contribute to the measured steady-state cellular levels of organic solutes. These include carrier-mediated and diffusional transport at both cellular membranes and intracellular compartmentation within vesicles or on macromolecules. As discussed previously for other solutes, our understanding of these mechanisms is still far from complete, and, without that understanding, we are not yet able to explain the observation that many compounds that apparently block luminal drug transport have little effect on steady-state cellular concentrations (16, 17, 26).

What is the nature of the luminal transporter for larger organic anions? The present data indicate that the transport of FL-MTX from cell to lumen is specific, concentrative, and dependent on cellular metabolism. At present, we do not know whether CSA, verapamil, or LTC4 inhibit FL-MTX transport from cell to lumen by competition for a single carrier protein or a family of related proteins. Thus it is not clear whether the luminal carrier(s) handles just anions or a mix of xenobiotics. It is unlikely, however, that the MDR transporter is involved, since this drug-transporting ATPase handles organic cations, weak organic bases, and some uncharged compounds but not organic anions such as LTC4 (6), and since, in killifish tubules, LTC4 has no effects on the transport of daunomycin, a fluorescent substrate for the MDR transporter (D. S. Miller, unpublished observations). A likely candidate would be a renal form of the hepatic canalicular (luminal) multispecific organic anion transporter (cMOAT), an ATPase that mediates the biliary excretion of large organic anions (21, 22). Recent data indicate that hepatic cMOAT is closely related to human MDR-associated protein (MRP) with nearly 50% sequence identity (23). Experiments with isolated canalicular membrane vesicles have shown that cMOAT exhibits a high affinity for LTC4 (as a substrate for transport and a competitive inhibitor; Ref. 11) and CSA (as a competitive inhibitor; Ref. 2). In that respect, the pattern of inhibition of cMOAT in hepatic membrane vesicles...
RENAL ORGANIC ANION TRANSPORT can be found in the present study for the cell-to-lumen transport of FL-MTX. Although verapamil has no effects on ATP-driven LTC$_4$ transport in hepatocyte canalicular membrane vesicles (11), there is suggestive evidence that it may inhibit MRP-mediated drug efflux in tumor cells (5). Verapamil sensitivity could represent one difference between the liver and kidney forms of related drug-transporting ATPases.

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