ORGANIC ANION TRANSPORT MECHANISMS IN RENAL TISSUE FROM Fundulus heteroclitus and Cancer borealis AS PROBED BY EPI-FLUORESCENCE MICROSCOPY and VIDEO IMAGE ANALYSIS

Daniel E. Bowen[1] and David S. Miller[2]
[1]Biology Department, Benedictine College, Atchison KS 66002
[2]Laboratory of Cellular and Molecular Pharmacology, NIH/NIEHS, Research Triangle Park, NC 27709

The renal proximal tubule plays an major role in the elimination of potentially toxic organic anions, e.g., metabolic wastes, drugs and environmental pollutants and drug and pollutant metabolites. This summer we used epi-fluorescence microscopy and video image analysis to investigate two aspects of the renal organic anion transport mechanism. The first is concerned with uptake at the basolateral membrane, the rate-determining step in secretion. Recent experiments show that organic anion uptake and net secretion can be increased by indirect coupling to the Na gradient (Pritchard and Miller, In: The Kidney: Physiology and Pathophysiology, Raven Press, 1992, pp 2921-2945). This occurs through two energetically coupled events at the basolateral membrane: monovalent-divalent organic anion exchange and Na-coupled divalent anion uptake. Importantly, in the absence of added divalent organic anions, monovalent organic anions are still actively accumulated. Is the same Na-dependent, indirect coupling mechanism used to drive basal transport?

To answer this question, we measured fluorescein (FL) uptake and efflux in proximal tubules from sea water adapted killifish, Fundulus heteroclitus. Recent studies have shown that FL is a substrate for the renal organic anion transport system in rabbit and teleost fish proximal tubules (Sullivan et al, Amer. J. Physiol. 258:F46-F51, 1990; Miller and Pritchard, Amer. J. Physiol. 261:R1470-R1477, 1991). Unlike other commonly studied substrates for transport, which can only be measured using chemical or radioisotopic means, FL is intensely fluorescent and its uptake into the cells and lumens of proximal tubules can be followed using a fluorescence microscope. Thus, use of this compound adds a new dimension to the study of organic anion transport, spatial resolution.

When killifish tubules were incubated in a buffered teleost saline solution (containing, in mM: 140 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$ and 20 TRIS, at pH 8.25) with 1 μM FL, the dye rapidly accumulated in the cells and lumen. Within 15 min, uptake in both compartments had reached a steady state. If 10 μM glutarate was present in the buffer from time-zero tubules showed roughly the same time course of uptake over 20 min, followed by a second uptake phase and a second higher plateau. Parallel patterns of uptake were seen in the tubular lumina where fluorescent intensity was 50-100% higher. Calibration of the imaging system using glass capillaries with known concentrations of FL indicated that steady state concentrations in the cells were about 25 μM at the first plateau and about 40 μM at the second plateau. Thus, even in the absence of added glutarate, cells accumulated FL to a level many times that of the medium. Glutarate caused a delayed increase in accumulation; the delay most likely represents the time needed to accumulate

sufficient glutarate to provide an additional driving force for anion exchange.

We have used LiCl as a tool to determine if basal, uphill accumulation of organic anions was driven by indirect coupling to Na. Lithium inhibits coupled organic anion transport by blocking Na-divalent organic anion uptake, but it does not affect anion exchange or disrupt cell metabolism (Pritchard, Amer. J. Physiol. 255:F597-F604, 1988; Miller, unpublished data). Two types of experiment showed that basal FL uptake is driven by indirect coupling to Na. First, when killifish proximal tubules were incubated with buffer containing 1 μM FL (no added glutarate) and increasing concentrations of LiCl, FL accumulation was inhibited in a dose dependent manner; with 20 mM LiCl, inhibition was nearly complete. Second, when tubules were loaded to steady state in buffer with 1 μM FL and then 20 mM LiCl added to the medium (without removing the FL), a net efflux of dye from the tissue was observed. The time course of efflux from the cells showed a 1-2 min delay followed by a single exponential with a mean half-time of 32 min (data from 5 tubules). In these efflux experiments, the buffer used contained no added divalent organic anions. Therefore, the most likely explanation for FL runout is that under basal conditions divalent organic anions, such as α-ketoglutarate (the major divalent organic anion in proximal tubule cells), are produced by intermediary metabolism and leak into the medium possibly in exchange for FL. Normally, these divalents anions are returned to the cells by the Na-dependent cotransporter and the steep divalent anion gradient (in>out) would drive FL uptake via exchange. When Li was added to the medium, divalent organic anion uptake was blocked, reducing the gradient for divalent anions. This, in turn, inhibited FL uptake and caused net loss of FL from loaded tubules.

The second question addressed concerns the distribution of organic anions within renal cells. We recently found that in crab urinary bladder (an invertebrate tissue with transport functions like vertebrate proximal tubule), intact teleost proximal tubules and monolayers of renal cells in culture, FL is distributed over two intracellular compartments; one is diffuse and cytoplasmic, the other is concentrated and vesicular (Miller et al, Bull. MDIBL 30:56-57, 1991; Miller et al, Am. J. Physiol., submitted). To determine if FL was trapped in vesicles as they formed at the plasma membrane or if the dye was taken up from the cytoplasm after transport into the cell, we incubated bladder slices from Cancer borealis in a crab Ringer's solution (containing, in mM: 449 NaCl, 11 KCl, 12.5 $CaCl_2$, 18.5 $MgCl_2$ and 50 TRIS, at pH 7.8) with FL and various effectors of organic anion transport and measured cytoplasmic and vesicular fluorescence at steady state (90-120 min). Incubation with 10 or 50 μM glutarate stimulated 0.5 μM FL uptake in cytoplasm and vesicles (Table 1). Uptake into the cytoplasm and vesicles was reduced by LiCl and the monovalent organic anions, probenecid and p-aminohippurate. Thus, treatments that stimulate transport at the basolateral membrane (low glutarate concentrations) stimulated vesicular uptake and treatments that inhibited transport (Li and competitor organic anions) reduced vesicular uptake. This indicates that vesicular uptake is dependent on the cytoplasmic FL concentration rather than the medium concentration. Thus, accumulation in vesicles is a two step process, involving uptake into the cytoplasm mediated by the basolateral transporter followed by transport into vesicles. This conclusion has been confirmed recently by microinjection experiments with

renal cells in culture. Moreover, those studies have shown that uptake by vesicles is concentrative, specific and energy dependent (Miller et al, Am. J. Physiol., submitted). The role that intracellular compartmentation plays in overall organic anion secretion remains to be determined.

Table 1. Effects of modifiers of organic anion transport on cytoplasmic and vesicular levels of fluorescein in C. borealis urinary bladder.

Treatment	Cytoplasm	Vesicles
Control	41 ± 1 (11)	64 ± 3
10 μM Glutarate	138 ± 5 (7)	212 ± 5
50 μM Glutarate	81 ± 3 (9)	107 ± 3
20 mM LiCl + 10 μM Glutarate	32 ± 2 (5)	NVD
1 mM Probenecid	27 ± 2 (6)	NVD
1 mM p-Aminohippurate	25 ± 2 (7)	NVD

Data given as mean ± SE pixel intensity (scale 0-255) for each intracellular region; the number of measurements is in parentheses. NVD, no vesicles detected (punctate areas of high fluorescent intensity were not seen). Because of the contribution of out of focus cytoplasmic fluorescence above and below vesicles and the small vesicle diameter (2-5 μm), measured vesicle intensities underestimate actual values by 5-40 fold.

Supported in part by a Pew Fellowship to D.E. Bowen.