ORGANIC ANION COMPARTMENTATION IN CRAB (CANCER BOREALIS) URINARY BLADDER CELLS ANALYZED BY CONFOCAL MICROSCOPY

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All animals possess a potent renal excretory transport system for potentially toxic, anionic metabolites, xenobiotics and xenobiotic metabolites. Over the past few years, it has become clear that the secretion of organic anions across renal epithelia is driven by tertiary active transport at the basolateral membrane (Pritchard and Miller, Am. J. Physiol., 261:R1329,1991). Although it had been assumed that organic anions cross the cell by simple diffusion, recent experiments suggest otherwise. We mapped the intracellular distribution of a fluorescent organic anion, fluorescein (FL), using epi-fluorescence microscopy and video imaging and found that in all organic anion secreting epithelia studied FL is distributed between two compartments: one diffuse and cytoplasmic, the other punctate (Miller and Pritchard, Am. J. Physiol., in press). The punctate compartment fills from the cytoplasm and microinjection studies show that uptake into that compartment is concentrative, specific and energy dependent.

This year, I studied 2 aspects of organic anion compartmentation: 1) FL distribution between the cytoplasmic and punctate compartments, and 2) the possibility that punctate sites of accumulation are mobile. A laser scanning, confocal microscope (Insight, Meridian Instruments) was used to optically section sheets of urinary bladder from a crab, Cancer borealis. This tissue is functionally analogous to vertebrate proximal tubule and the mechanisms that drive organic anion secretion across proximal tubule and crab bladder are identical (Miller et al, Am. J. Physiol., 257:R501,1989). Tissue was loaded with FL in vitro by incubation of bladder sheets or in vivo by injecting the animal with 1 ml of 1 mM FL in a crab Ringers (CR) 24 h before sacrifice. Preliminary experiments established that after FL was injected into crabs, the dye rapidly concentrated in bladder urine, with urine to hemolymph concentration ratios (U_{FL}/H_{FL}) exceeding 100; in contrast, U/H for filtration markers do not exceed 2 in this species (Holliday and Miller, Am. J. Physiol., 246:R364,1984). Fluorescein excretion into the sea water approximated a single exponential with a half time ($T_{1/2}$) of 2 d. Adding 10 mM p-aminohippurate (PAH), a model substrate for the renal organic anion transport system, to the injection solution elevated H_{FL}, reduced U_{FL} and increased the $T_{1/2}$ of excretion to 5.5 d. These findings are consistent with a common, renal route of excretion for PAH and FL, the organic anion system.

Only minor differences in dye distribution were seen when confocal micrographs of bladder tissue loaded with FL in vitro and in vivo were compared. In both cases, the overall pattern was of discrete, intensely fluorescent vesicles embedded in a less intensely labeled cytoplasm. The mean diameter for well-resolved, circular sites was 3.1 ± 0.2 μm (SE; range 0.9-9.0 μm; 112 measurements, tissue from 4 crabs) for tissue loaded in vitro and 3.8 ± 0.3 μm (range 0.6-10.3 μm; 89 measurements, tissue from 4 crabs) for tissue loaded in vivo. In two experiments where bladder tissue was loaded by 1.5 h incubation in CR with 5 μM FL, 33-53% of total tissue fluorescence was found in the punctate compartment (Table 1); in one experiment with in vivo

loading, the corresponding value was 60 ± 7%. In vitro treatments that are known to alter FL uptake at the basolateral membrane of bladder cells, e.g., exposure to PAH or glutarate, had the expected effect on cytoplasmic fluorescence (Table 1). PAH and the low concentration of glutarate also changed the distribution of the fluorescence between the two compartments. Exposing bladder tissue to nocodazole, a drug that depolymerizes microtubules, had no effect on cytoplasmic fluorescence, but significantly reduced punctate fluorescence (Table 1).

To determine whether punctate sites of FL accumulation are mobile, I measured the displacement of the confocal microscope's fine focus knob needed to keep 2-3 μm diameter sites in sharp focus. For each vesicle, measurements were made over 4-5 min and an average rate calculated. Out of 35 fluorescent vesicles studied in bladder tissue from 4 crabs (3 loaded in vitro, 1 in vivo) all but one was seen to move in the basal-to-apical, i.e., secretory, direction. The mean rate of movement was 0.83 ± 0.11 μm/min. Exposing bladder tissue to 9 or 18 μM nocodazole reduced the rate of vesicle movement by 70-80%. Since nocodazole had no effect on the cytoplasmic accumulation of FL, a process dependent on metabolic energy and the Na,K-ATPase (Table 1), the drug's effects on vesicle movement likely resulted from cytoskeletal disruption rather than general toxicity.

In summary, the present data show that 1) organic anions are compartmentalized within crab bladder cells in vivo as well as in vitro, 2) about half of intracellular FL is in the vesicular compartment, and 3) FL containing vesicles move through bladder cells in the secretory direction by a microtubule-dependent mechanism. (I thank Meridian Instruments for the loan of the confocal microscope and Mark Gaucher, from Meridian, for help with the instrument.)

Table 1. Compartmentation of Fluorescein (FL) in C. Borealis Urinary Bladder Cells

Treatment	Cytoplasmic Fluorescence	% Fluorescence in Vesicles
Control	93 ± 7	53 ± 7
PAH, 1 mM	20 ± 3	19 ± 8
Glutarate, 50 μM	134 ± 7	45 ± 1
Glutarate, 500μM	14 ± 1	None Detected
Control	42 ± 7	33 ± 3
Nocodazole, 9 μM	41 ± 5	19 ± 6
Nocodazole, 18 μM	43 ± 2	2 ± 1

Shown are results of 2 experiments in which sheets of bladder tissue were incubated in CR containing 5 μM FL and the drugs indicated. After 1.5 h, tissue was removed to a viewing chamber on the stage of the confocal microscope fitted with a video camera connected to a MacIntosh computer.Images were stored on optical disk. For analysis, images were recalled and segmented into areas of diffuse and punctate fluorescence. For each compartment, average pixel intensity, area and total fluorescence were measured. Data are given as mean ± SE for 10-12 measurements from 4 bladder sheets; cytoplasmic fluorescence is expressed as mean pixel intensity.