p-GLYCOPROTEIN-MEDIATED SECRETION OF A FLUORESCENT CYCLOSPORIN ANALOGUE BY KILLIFISH (FUNDULUS HETEROCLITUS) RENAL PROXIMAL TUBULES

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Certain cultured cells develop resistance to multiple therapeutic agents by expression of one or more multidrug resistance (MDR) genes. These code for plasma membrane ATPases (called MDR transporters, p-glycoprotein or gp-170) that mediate the active efflux of a wide variety of hydrophobic drugs from the cells (Ford and Hait, Pharmacol. Rev. 42:155-199, 1990). MDR genes are also expressed in several normal tissues (Fojo et al., PNAS 84:265-269, 1987; Thiebaut et al., PNAS 84:7735-7738, 1987). In kidney, the apical membrane of the proximal tubule epithelium is particularly rich in p-glycoprotein, placing this pump in the correct location to mediate the excretion of xenobiotics. Consistent with a role for p-glycoprotein as an excretory transporter, monolayers of LLC-PK1 and OK cells, cell lines that express many properties of proximal tubule cells, exhibit net secretion of several MDR substrates, including, vinblastine, verapamil, cyclosporin A (CsA) and digoxin (Horio et al., Biochim. Biophys. Acta 1027:116-122, 1990; Ueda et al., J. Biol. Chem. 267:24249-24252, 1992; Saeki et al., J. Biol. Chem. 269:6077-6080, 1993). However, the role of p-glycoprotein in the excretion of xenobiotics by intact proximal tubules has not been directly addressed.

In the present study we used epi-fluorescence microscopy and digital image analysis to study the transport of a fluorescent CsA derivative ([N-ε(4-nitrobenzofurazan-7-yl)-D-Lys^8]-cyclosporin; NBD-CsA; Sandoz Pharma) in intact renal proximal tubules from killifish. CsA is a lipophilic, undecapeptide with potent immunosuppressant activity. In addition, CsA reverses drug resistance in malignant cancer cell models, primarily by inhibiting drug efflux through p-glycoprotein (Twentyman, Biochem. Pharmacol. 43:109-117, 1992). It is also a substrate for transport by p-glycoprotein in cultured cells (Ford and Hait, op. cit.; Saeki et al, op.cit.). Unlike many MDR substrates, CsA is neither an organic cation nor a weak organic base and thus, should not be a substrate for transport by the renal organic cation/proton exchanger.

For experiments, killifish renal tubular masses were isolated in a marine teleost saline (containing, in mM:140 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$ and 20 tris(hydroxymethyl)-amino methane, at pH 8.25). Under a dissecting microscope each mass was teased with fine forceps to remove adherent hematopoietic tissue. Individual killifish proximal tubules were dissected free of the masses and transferred to a foil-covered Teflon chamber (Bionique) containing 1 ml of marine teleost saline with NBD-CsA and added effectors. The chamber floor was a 4x4 cm glass cover slip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted microscope equipped with epi-fluorescence optics (fluorescein filter cube) and a video camera and Macintosh computer (Miller et al, Amer. J. Physiol. 264:R882-R890, 1993). Cyclosporins were added to the medium in dimethylsulfoxide (DMSO) stock solutions. Preliminary experiments showed that the concentrations of DMSO used (0.05-0.5%) had no significant effects on the uptake and distribution of NBD-CsA or fluorescein as measured by epi-fluorescence microscopy. All experiments were conducted at 18-20° C.

Tubules incubated in medium containing 0.5 μM NBD-CsA exhibited rapid increases in fluorescence intensity in both the cellular and lumenal compartments (Fig. 1A). At all but the earliest times, luminal fluorescence exceeded cellular by a factor of 2-3. Addition of 5 μM CsA to the medium had no effect on cellular fluorescence, but greatly reduced luminal fluorescence; with CsA in the bath, fluorescence intensities in the two regions were equal at all times (Fig. 1B). This

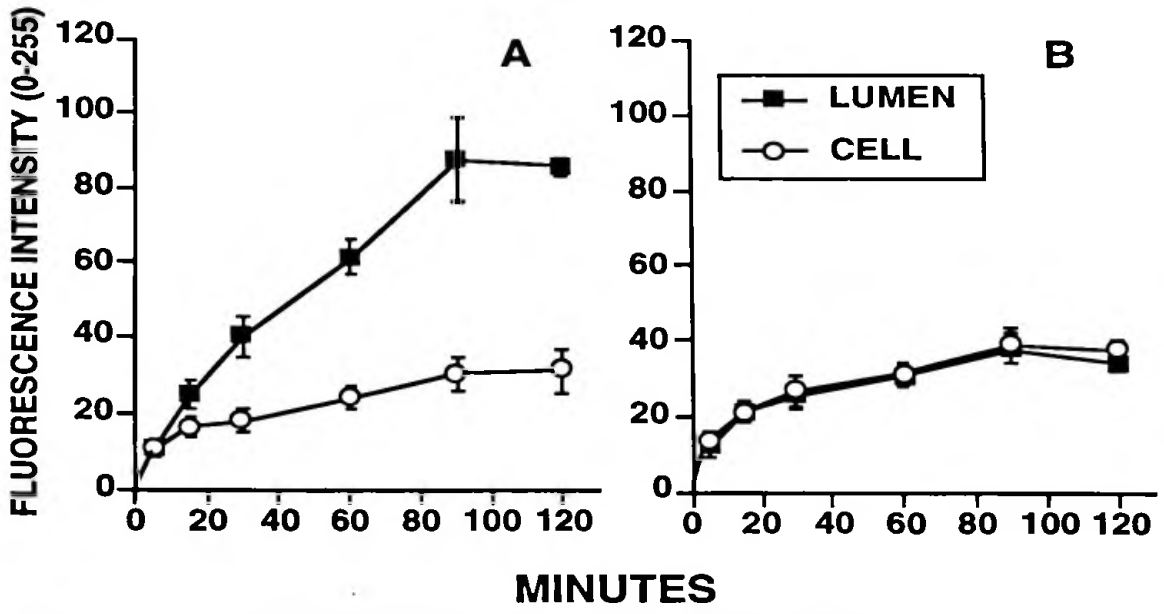

Figure 1. Time course of 0.5 μM NBD-CsA accumulation in killifish renal proximal tubules. Tubules were incubated in media without (control, A) or with 5 μM CsA (B). Each point shows the mean fluorescent intensity in cells and lumens for 5-9 tubules; variability is given by SEM bars. CsA had no significant effects on cellular fluorescence. At all times except 5 min, CsA significantly reduced luminal fluorescence ($P<0.01$).

inhibitory effect of CsA was not due to toxicity, since exposing tubules to 5 μM CsA for 1-2 h had no effects on the energy-dependent and concentrative accumulation of the fluorescent organic anion, fluorescein (not shown). This finding also suggests that the plateau in luminal NBD-CsA accumulation seen after 90 min of incubation with 0.5 μM substrate (Fig. 1A) is not a result of cyclosporin-induced nephrotoxicity.

Table 1 shows that luminal accumulation of the dye was abolished by CsA, verapamil, vinblastine and quinine, all substrates for p-glycoprotein. Luminal dye accumulation was also reduced by vanadate, a potent inhibitor of p-glycoprotein ATPase activity, and by the metabolic poisons, dinitrophenol and KCN. Tetraethylammonium and p-aminohippurate, substrates for the renal organic cation and anion transport systems, respectively, had no effects on dye accumulation. None of these treatments had any significant effect on cellular NBD-CsA accumulation (Table 1).

Recent studies indicate that teleost fish possess p-glycoprotein genes (Chen et al., Biochim. Biophys. Acta 1171:65-72, 1992) and that these are expressed in at least one transporting epithelium, killifish opercular skin (Karnaky et. al., Bull. MDIBL 32:61-62, 1993). The present results provide evidence that another killifish epithelium, renal proximal tubule, also expresses this transport ATPase. Our data indicate that NBD-CsA entered proximal tubule cells by simple diffusion, but was transported from cell to tubular lumen by a process that was saturable (not shown), concentrative, dependent on cellular metabolism and inhibited by CsA, other p-glycoprotein substrates and vanadate. Substrates for the renal organic anion or organic cation transport systems did not inhibit. Thus, it appears that NBD-CsA is actively secreted into the tubular lumen of proximal tubules by the MDR transporter. To our knowledge, the present report is the first demonstration that p-glycoprotein mediates the secretion of any drug in intact proximal

tubules. Since many drugs handled by the classical organic cation transport system are also substrates for p-glycoprotein (Pritchard and Miller, Physiol. Rev. 73:765-796, 1993), one would expect their transport from cell to lumen to be mediated by both systems. CsA could prove to be a useful tool in sorting out the contributions of the two lumenal transporters. Also, the therapeutic usefulness of CsA is limited by nephrotoxicity, with both glomerular and tubular functions affected (Mason, Pediat. Nephrol. 4:554-574, 1992). The high lumenal concentrations generated by p-glycoprotein in proximal tubule may contribute to the toxic effects of the drug.

Table 1. Effects of transport inhibitors on NBD-CsA accumulation by killifish proximal tubules.

Treatment	Fluorescence Intensity		Lumen/Cell
	Cell	Lumen	
Control(11)	27.0 ± 2.2	76.5 ± 4.7	2.9 ± 0.2
50 μM Verapamil (9)	32.0 ± 4.2	33.3 ± 4.5**	1.1 ± 0.1**
50 μM Vinblastine (9)	33.0 ± 4.5	44.6 ± 5.2**	1.4 ± 0.1**
1 mM DNP (5)	38.2 ± 6.0	43.0 ± 4.6**	1.2 ± 0.1**
1 mM TEA (6)	34.7 ± 7.4	86.5 ± 9.1	2.9 ± 0.5
Control(8)	32.5 ± 3.3	69.3 ± 5.8	2.2 ± 0.2
5 μM Verapamil (8)	43.6 ± 7.4	54.6 ± 11.0	1.3 ± 0.1**
50 μM Verapamil (7)	35.3 ± 4.4	37.7 ± 4.5**	1.1 ± 0.1**
Control (9)	53.9 ± 6.1	120.4 ± 13.6	2.3 ± 0.2
1 mM KCN (5)	50.4 ± 6.1	61.6 ± 9.9**	1.2 ± 0.1**
Control (7)	76.4 ± 6.7	144.7 ± 15.9	1.9 ± 0.2
1 mM PAH (6)	72.3 ± 4.7	139.5 ± 8.0	2.0 ± 0.2
Control (11)	28.4 ± 3.7	67.3 ± 8.4	2.5 ± 0.2
5 μM CsA (8)	30.0 ± 2.2	31.0 ± 3.9**	1.0 ± 0.1**
50 μM Quinine (7)	22.9 ± 3.0	38.9 ± 6.9*	1.3 ± 0.2**
Control (7)	47.3 ± 5.8	90.6 ± 6.7	2.0 ± 0.2
50 μM VO_3	39.3 ± 4.1	36.5 ± 3.1**	0.9 ± 0.1**

Tubules were incubated in medium containing 0.5 μM NBD-CsA without (control) and with the indicated chemicals. After 60 min, fluorescent images were acquired and analyzed. Data given as mean ± SE with the number of tubules in parentheses. Abbreviations: DNP, dinitrophenol; TEA, tetraethylammonium; PAH, p-aminohippurate. Statistical comparisons: * significantly lower than controls, $P<0.05$; ** significantly lower than controls, $P<0.01$.

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