Transport of a fluorescent cAMP analog in teleost proximal tubules

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Reichel V, Masereeuw R, van den Heuvel JJ, Miller DS, Fricker G. Transport of a fluorescent cAMP analog in teleost proximal tubules. Am J Physiol Regul Integr Comp Physiol 293: R2382–R2389, 2007. First published September 12, 2007; doi:10.1152/ajpregu.00029.2007.—Previous studies have shown that killifish (Fundulus heteroclitus) renal proximal tubules express a luminal membrane transporter that is functionally and immunologically analogous to the mammalian multidrug resistance-associated protein isoform 2 (Mrp2, ABCC2). Here we used confocal microscopy to investigate in killifish tubules the transport of a fluorescent cAMP analog (fluoro-cAMP), a putative substrate for Mrp2 and Mrp4 (ABCC4). Steady-state luminal accumulation of fluoro-cAMP was concentration, specific, and metabolism-dependent, but not reduced by high K+ medium or ouabain. Transport was not affected by p-aminohippurate (organic anion transporter inhibitor) or p-glycoprotein inhibitor (PSC833), but cell-to-lumen transport was reduced in a concentration-dependent manner by Mrp inhibitor MK571, leukotriene C4 (LTC4), azidothymidine (AZT), cAMP, and adefovir; the latter two compounds are Mrp4 substrates. Although MK571 and LTC4 reduced transport of the Mrp2 substrate fluorescein-methotrexate (FL-MTX), neither cAMP, adefovir, nor AZT affected FL-MTX transport. Fluoro-cAMP transport was not reduced when tubules were exposed to endothelin-1, Na nitroprusside (an nitric oxide generator) or phorbol ester (PKC activator), all of which signal substantial reductions in cell-to-lumen FL-MTX transport. Fluoro-cAMP transport was reduced by forskolin, and this reduction was blocked by the PKA inhibitor H-89. Finally, in membrane vesicles from Spodoptera frugiperda (SF9) cells containing human MRP4, ATP-dependent and specific uptake of fluoro-cAMP could be demonstrated. Thus, based on inhibitor specificity and regulatory signaling, cell-to-lumen transport of fluoro-cAMP in killifish renal tubules is mediated by a transporter distinct from Mrp2, presumably a teleost form of Mrp4.

THE VERTEBRATE RENAL PROXIMAL TUBULE is responsible for the active excretion of potentially toxic, negatively charged metabolic wastes, xenobiotics, and xenobiotic metabolites. To accomplish this task, this epithelium is polarized with the basolateral and luminal membranes containing a broad variety of organic anion transport proteins, including members of the multidrug resistance-associated protein (MRP), organic anion transporter (OAT), and organic anion transporting polypeptide (OATP) families. The MRPs, a subfamily of the ATP binding cassette protein superfamily, comprise at least nine members (MRP1–9). All are membrane-bound proteins and depend on ATP hydrolysis to drive substrate transport. In mammalian kidney, MRPs are located on the apical membrane of proximal tubular epithelial cells and play an important role in the urinary excretion of a wide variety of compounds. Substrates for MRPs in rat and man include glutathione, glucuronide, and sulfate conjugates. MRP2 also mediates transport of leukotriene C4 (LTC4), estradiol-17b-glucuronide, p-aminohippurate (PAH), S-glutathionyl 2,4-dinitrobenzene, and anticancer substances like methotrexate and anthracyclines and indirubins.

For a functional assessment of organic anion transport in the kidney we have used isolated killifish (Fundulus heteroclitus) renal proximal tubules. Kidneys of this teleost fish contain a high fraction of proximal tubules, which can be isolated easily and are viable for at least a day at room temperature. This comparative model is a powerful tool for the study of secretory transport in intact proximal tubules. Unlike mammalian proximal tubules, the broken ends of these tubules reseal after isolation, thus forming a closed fluid-filled luminal compartment. This enables study of cellular uptake and luminal accumulation of fluorescent compounds using imaging techniques.

We previously reported on the function and regulation of an MRP2-like protein (7, 8, 17, 18) and the immunolocalization of a MRP4-like protein (19) in killifish proximal tubules. Consistent with this, an MRP4-like gene has been identified in the genome of zebrafish (Danio rerio), another teleost fish [European Molecular Biology Laboratory (EMBL) accession no. AL591370.1]. In the present study, we used confocal microscopy and the fluorescent cAMP analog fluoro-cAMP to visualize transport across proximal tubular epithelium of the killifish. Our data indicate that tubular transport of fluoro-cAMP is distinct from MRP2-mediated transport and presumably mediated by a teleost form of Mrp4.

MATERIALS AND METHODS

**Chemicals.** Fluoro-cAMP was purchased from Biolog Life Science Institute (San Diego, CA and Bremen, Germany). Fluorescein-methotrexate (FL-MTX) and phorbol 12-myristate 13-acetate were obtained from Sigma-Aldrich and Oligo (Valleymount, CA). PSC833 and H-89 were obtained from Calbiochem (San Diego, CA and Bremen, Germany). MK571 and LTC4 were obtained from Tocris (Ellisville, MO). Adefovir and forskolin were obtained from Sigma-Aldrich and Research Biochemicals (Natick, MA), respectively. ATP, ADP, and cAMP were obtained from ThermoFisher Scientific (Waltham, MA). Other chemicals were purchased from Sigma-Aldrich.

**Animals.** All animals were handled according to the guidelines of the Animal Care and Use Committee of the University of Heidelberg.

**Confocal microscopy.** Tubules obtained from killifish kidneys were washed by centrifugation (1000 g, 5 min) and stained with 0.5 μM fluoro-cAMP (Molecular Probes, Eugene, OR) for 10 min at room temperature. After washing, confocal microscopy was performed with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) equipped with a 63×/1.4 oil immersion objective (Nikor, Tokyo). Imaging was performed using the Leica LAS software (version 2.5.0.613). Excitation was provided by 488-nm laser lines, and the emission was collected at 500 to 560 nm. For each sample, 20 optical sections were collected. The optical sections were imported into ImageJ and analyzed for the cumulative fluorescence intensity (FI).

**Immunocytochemistry.** Tubules were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.1% Triton X-100, tubules were incubated with primary antibodies against Mrp4 (ABCC4, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and Mrp2 (ABCC2, 1:100; Dianova, Hamburg, Germany) for 1 h at room temperature. After washing, tubules were incubated with a 1:200 dilution of Alexa Fluor 488 (Invitrogen, Carlsbad, CA)-conjugated secondary antibodies for 1 h at room temperature. Images were acquired with a fluorescence microscope (Eclipse TE2000 U; Nikon, Tokyo, Japan) equipped with an AxioCam MRc (Carl Zeiss, Jena, Germany). The optical sections were imported into ImageJ and analyzed for the cumulative FI.

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tained from Molecular Probes (Eugene, OR). Valspodar (PSC383) was from Novartis (Basle, Switzerland). All other chemicals were obtained from Sigma (St. Louis, MO; Zwijndrecht, the Netherlands). Primary Mrp4 antibody was a human Mrp4 antibody (pAb rM4-pl) raised in rabbit at the Nijmegen Centre for Molecular Life Sciences (Nijmegen, The Netherlands) as described in Van Aubel et al. (31). For this antibody, the linker region (amino acids 611–676) of human MRP4 was chosen as epitope for the polyclonal antibody we used in this study. We aligned human and zebrafish (AL591370) Mrp4 sequences and found 68% homology. Within the linker region (amino acids 611–676) we found 45% homology; at least 6 amino acids in line are conserved. This should be sufficient for some cross-reactivity. Goat-derived anti-rabbit IgG 488 was obtained from Molecular Probes.

Animals and tissue preparation. All animal studies were performed in accordance with institutional regulations for animal protection and had been approved by the animal care and use committee of Mount Desert Island Biological Laboratory. Killifish were collected in the vicinity of Mount Desert Island, Maine. Fish were held in tanks with natural flowing sea water at the Mount Desert Island Biological Laboratory. After death, masses of renal tubules were extracted and transferred to foil-covered Teflon chambers (Bionique) with glass coverslip bottoms. The chamber contained 1 ml of marine teleost solution without (controls) or with added chemicals. These were added as stock solutions in marine teleost saline or in DMSO. The final DMSO concentration did not exceed 0.5%. This was from Novartis (Basle, Switzerland). All other chemicals were obtained in the presence of 5&AMP. At indicated time points, samples were taken and diluted in 900 μl ice-cold TS buffer to stop transport. Samples were filtered through NC45 filters (Whatman, Maidstone, UK) on a sampling manifold (Millipore, Billerica, MA). Filters were extracted in SDS/HPEES buffer (1% SDS, 7.5 mM HEPES) for at least 30 min. Afterward fluid fluorescence was measured using a Shimadzu RF-5301PC spectrofluorophotometer (excitation: 480 nm, emission: 520 nm). Experiments were performed in triplicate.

ATP-dependent transport was calculated by subtracting values obtained in the presence of 5&AMP from those obtained in the presence of ATP. To calculate MRP4-specific transport, respectively, values for ATP-dependent transport of control vesicles were subtracted from those of infected cell membrane vesicles.

Statistics. Values are presented as means ± SE. Means of control and treated groups were compared using one-way ANOVA and Dunnett’s post hoc test. Differences were considered statistically significant when P < 0.05.

RESULTS

Figure 1 shows a typical confocal image of a control killifish tubule after 30 min (steady-state) incubation in medium containing 2 μM fluo-cAMP. The distribution of fluorescence is similar to that seen for other fluorescent organic anions, i.e., the Mrp2-substrate FL-MTX and the Oat substrate, fluorescein (13, 16). That is, fluorescence intensity in the lumens > cells > medium. This pattern suggests a two-step process involving uptake at the basolateral membrane and efflux into the lumen.

Quantitation of confocal images showed that luminal accumulation of fluo-cAMP increased rapidly over the first 20 min and reached a steady-state level within 25–30 min (Fig. 2A). Cellular fluorescence remained constant from 10 min on. At steady state, luminal fluorescence exceeded cellular fluores-
cence by a factor of 5–8. Steady-state luminal fluo-cAMP accumulation was reduced when cellular energy metabolism was inhibited by 1 mM NaCN (Fig. 2B). In contrast, cellular fluorescence was not affected by NaCN. Exposing tubules to 1 mM ouabain did not affect steady-state fluo-cAMP accumulation (Fig. 2C). In killifish tubules, this concentration of ouabain abolishes Na-dependent transport of the fluorescent small organic anion, fluorescein (13). Similarly, increasing medium K\(^+\) by a factor of 10 was without effect (Fig. 2D); this treatment depolarizes killifish renal epithelial cells by about 40 mV and substantially inhibits uptake of organic cations (26). These results indicate that concentrative fluo-cAMP transport from cell to lumen was energy-dependent but not sensitive to changes in ion gradients or the membrane electrical potential.

Fig. 3. Inhibition of fluo-cAMP transport. Killifish tubules were incubated for 30 min in medium containing 2 μM fluo-cAMP without (control) or with the indicated additions. MK571, Mrp inhibitor; LTC\(_4\), leukotriene C\(_4\); PAH, p-aminohippurate; PSC833, p-glycoprotein inhibitor. Images were collected and analyzed as described in MATERIALS AND METHODS. Data are presented as the mean values for 12 tubules; variability is shown as SE bars. **Significantly different from controls, \(P < 0.01\).
To determine the characteristics of cellular and luminal accumulation of fluo-cAMP, we examined the effects of a number of chemicals on tubular transport. These chemicals were chosen because of known interactions with specific transporters or groups of related transporters. Previous studies with killifish tubules showed that cell-to-lumen transport of the Mrp2 substrate, FL-MTX, was sensitive to inhibition by MK571 and LTC₄, both inhibitors of MRPs (6, 11). Figure 3, A and B shows that MK571 and LTC₄ reduced luminal accumulation of fluo-cAMP in a concentration-dependent manner; neither compound affected cellular accumulation of fluo-cAMP. In contrast, the inhibitor of transport mediated by Oat, PAH, and the p-glycoprotein inhibitor (PSC833) were without effect (Fig. 3, C and D), even at concentrations known to block Oat-mediated transport of fluorescein and p-glycoprotein-mediated transport of a fluorescent CSA derivative, respectively, in killifish tubules (15, 23).

The MRP4 substrates, cAMP and adefovir (PMEA) caused concentration-dependent inhibition of steady-state luminal accumulation of fluo-cAMP with no change in cellular accumulation (Fig. 4). The same was observed with azidothymidine.
(AZT), which inhibits MRP4 in its monophosphate form (12, 21).

None of these latter compounds affected transport of FL-MTX (Fig. 4), indicating that they do not interact with teleost Mrp2. These results indicate that cell-to-lumen transport of fluo-cAMP and FL-MTX are mediated by different transporters, likely different teleost Mrp isoforms.

The stable cGMP analog, 8-bromo-cGMP, also decreased luminal accumulation of fluo-cAMP (Fig. 4). 8-Bromo-cGMP inhibits cAMP-specific phosphodiesterases, causing intracellular cAMP levels to increase. As a consequence, excretion of fluo-cAMP was inhibited.

Masereeuw and colleagues (13, 14) showed that cell-to-lumen transport of FL-MTX in killifish proximal tubules is mediated by a teleost homolog of Mrp2. Activity of this transporter is regulated by a signaling pathway involving endothelin-1 (ET-1) signaling through an endothelin B (ETB) receptor, nitric oxide synthase (NOS), and PKC (14, 17). Activating this pathway by exposing killifish tubules to 1–100 nM ET-1, 10–100 μM Na nitroprusside (an NO donor), or 1–100 nM phorbol ester (activates PKC) substantially reduces luminal accumulation of FL-MTX. Figure 5 shows that neither ET-1, Na nitroprusside, nor phorbol-12-myristate-13-acetate had any effect on the transport of fluo-cAMP in killifish tubules.

FL-MTX transport in killifish tubules is not affected by forskolin, an indirect activator of PKA (14). Figure 6 shows that 10 μM forskolin substantially reduced steady-state luminal accumulation of fluo-cAMP. This effect was blocked by the PKA inhibitor H-89, which by itself did not affect fluo-cAMP transport.

To determine whether fluo-cAMP was a substrate for human MRP4, transport studies were performed using membrane vesicles isolated from Sf9 cells transfected with human MRP4. We found ATP-dependent and specific uptake of fluo-cAMP into MRP4 expressing vesicles. The time course study showed that uptake of 20 μM fluo-cAMP in Sf9-MRP4 membrane vesicles started to reach a plateau after 20–30 min (Fig. 7A). Uptake clearly saturated with increasing fluo-cAMP concentration (Fig. 7B); apparent K_{\text{m}} and V_{\text{max}} values were 5.3 ± 2.0 μM and 1.1 ± 0.2 nmol·mg^{-1}·min^{-1}, respectively. Both MK571 and LTC4 caused concentration-dependent inhibition of fluo-cAMP uptake in vesicles from MRP4-infected cells (Figs. 7, C and D).

**DISCUSSION**

With the use of teleost renal tubules, fluorescent substrates and confocal microscopy can be applied to both visualize and measure all steps in the excretory pathway. For organic anions, previous imaging studies with killifish tubules have characterized an Oat-like uptake transporter on the basolateral membrane and an Mrp2-like efflux transporter on the luminal membrane. The basolateral teleost transporter drives the concentrative uptake of small organic anions (PAH, 2,4-D and fluorescein, not FL-MTX) through indirect coupling to the Na⁺ gradient. It is functionally analogous to mammalian Oat1 and Oat3 and a recent report suggests that the mammalian Oat1/Oat3 dichotomy is a result of an early gene duplication in lower vertebrates (1). The efflux transporter in killifish tubules drives concentrative accumulation of larger organic anions (FL-MTX, Texas Red, not fluorescein or PAH) into the tubular lumen (13). This uphill step is Na⁺-dependent, ouabain insensitive, and insensitive to changes in membrane electrical potential, suggesting that it is ATP-driven.

Both the Oat-like and Mrp2-like transporters appear to be regulated by PKC-dependent signals, with PKC activation rapidly (minutes) reducing transport for both (14, 16). In this regard, PKC activation is known to reduce Na⁺-dependent organic anion uptake in mammalian renal slices and tubules and a number of mammalian renal cell lines as well as in killifish tubules (16, 29, 34). Experiments with *Xenopus* oocytes expressing hOAT1 or flOat show both reduced specific transport and transporter internalization following PKC activa-

Fig. 5. Lack of effect of endothelin (ET), Na nitroprusside, and phorbol-12-myristate-13-acetate (PMA) on fluo-cAMP transport in killifish tubules. Tubules were incubated for 30 min in medium containing 2 μM fluo-cAMP without (control) the indicated additions. Data are presented as the mean values for 10–15 tubules; variability is shown as SE bars.
tion (9, 34). For the efflux transporter, both ET-1 and calcitropic hormones act through PKC to reduce transport (14, 17, 33).

Previous imaging studies with killifish tubules have suggested that there are additional OATs at the luminal membrane. First, Miller et al. (15) presented evidence for a potential-insensitive efflux pathway for fluorescein, carboxyfluorescein and bimane-S-conjugates. This pathway was sensitive to inhibition by PAH and it was assumed to be the major efflux step for small organic anions; it has not been investigated further. Second, Notenboom et al. (19) recently demonstrated that an antibody raised against human MRP4 immunolocalized to the luminal membrane of killifish renal proximal tubules. In that study, prolonged exposure (12–24 h) of tubules to gentamicin or ET-1 upregulated FL-MTX transport into the tubular lumen and increased luminal membrane immunoreactivity to an antibody raised to rabbit Mrp2. No changes in Mrp4 immunoreactivity were seen, suggesting the presence of a second Mrp isoform in the luminal membrane. Consistent with this, recent mining of the zebrafish genome indicated the presence of a sequence coding for a teleost Mrp4 homolog (EMBL accession no. AL591370.10).

Taken together, the results of the present study are consistent with the involvement of a teleost Mrp4 in the cell-to-lumen transport of fluo-cAMP. Measurements of steady-state fluo-cAMP accumulation in tubules suggested two concentrative steps, one at the basolateral membrane and the other at the luminal membrane. However, no treatment used (NaCN, ouabain and high medium K⁺, competitor organic anions) had any effect on cellular fluo-cAMP accumulation. Thus, it is unlikely that the uptake step is mediated and cellular accumulation must reflect simple diffusion plus binding of the dye to cellular components.

This was not the case for luminal accumulation of fluo-cAMP, which was substantially reduced when metabolism was inhibited by NaCN and when a number of organic anions were added to the medium. Clearly, cell-to-lumen transport was mediated and directly or indirectly coupled to ATP. This step was not affected by ouabain or high K⁺, suggesting that it was independent of ion gradients and membrane electrical potential. Luminal accumulation was not affected by millimolar
concentrations of PAH nor by micromolar concentrations of PSC833, ruling out involvement of the efflux pathway for small organic anions (15) and γ-glucoprotein. Luminal accumulation was reduced by MK571, LTC4, cAMP, PMEA, and AZT. Although MK571 and LTC4 have been shown to reduce luminal accumulation of FL-MTX in killifish tubules (14), neither cAMP, PMEA, nor AZT had any effect on FL-MTX transport (present study). Finally, luminal accumulation of fluo-cAMP was not affected when ET-1/NOS/PKC signaling was activated (present study); this signaling pathway reduces luminal accumulation of FL-MTX (14, 17). Fluo-cAMP transport was reduced by PKA activation (present study), which does not affect FL-MTX transport (14).

Thus, based on inhibitor specificity and regulatory mechanisms, cell-to-lumen transport of FL-MTX and fluo-cAMP appear to occur through separate and distinct luminal membrane transporters. For fluo-cAMP, sensitivity of transport to cAMP, PMEA, and AZT suggests involvement of a teleost MRP4. Consistent with this, fluo-cAMP proved to be a substrate for transport by human MRP4 with a 10-fold higher apparent affinity than cAMP (present study and Ref. 2). Thus, it is likely that a teleost MRP4 mediates cell-to-lumen transport of fluo-cAMP in killifish renal tubules.

Perspectives and Significance

Isolated teleost renal proximal tubules provide an important comparative model for the study of renal excretory transport. The present study gives functional evidence for a teleost isoform of MRP4 and suggests that MRP4 isoforms may have a regulatory impact on cellular nucleotides. In addition, the study demonstrates that fluo-cAMP may be a useful tool to investigate MRP4 regulation and function.

GRANTS

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