Multidrug resistance protein Mrp2 mediates ATP-dependent transport of classic renal organic anion p-aminohippurate

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Van Aubel, Rémon A. M. H., Janny G. P. Peters, Rosalinde Masereeuw, Carel H. van Os, and Frans G. M. Russel. Multidrug resistance protein Mrp2 mediates ATP-dependent transport of classic renal organic anion p-aminohippurate. Am J Physiol Renal Physiol 279: F713–F717, 2000.—p-Aminohippurate (PAH) is widely used as a model substrate to characterize organic anion transport in kidney proximal tubules. The carrier responsible for uptake of PAH across the basolateral membrane has been cloned and well characterized, whereas transporters mediating PAH excretion across the brush-border (apical) membrane are yet unknown. In this study we investigated whether PAH is a substrate for the apical multidrug resistance protein 2 (Mrp2). Overexpression of recombinant rabbit Mrp2 in Sf9 cells significantly increased ATP-dependent [14C]PAH uptake into isolated membrane vesicles compared with endogenous ATP-dependent uptake. The Michaelis-Menten constant and maximal velocity for PAH-mediated ATP-dependent [14C]PAH transport were 1.9 ± 0.8 mM and 187 ± 29 pmol·mg⁻¹·min⁻¹, respectively. On the basis of the inhibitory profile, the endogenous ATP-dependent PAH transporter does not appear to be an ortholog of Mrp2. Together, our results show that Mrp2 is a low-affinity ATP-dependent PAH transporter, indicating that Mrp2 might contribute to urinary PAH excretion.

The kidney plays an important role in the clearance of endogenous compounds and xenobiotics from the body. The proximal tubule is the primary site at the nephron where organic anions are taken up from the blood and excreted into urine. p-Aminohippurate (PAH) is widely used as a model substrate to investigate renal handling of organic anions because of its high renal clearance and low susceptibility to metabolism. Uptake of PAH from the blood across the basolateral membrane is mediated by the PAH/dicarboxylate exchanger OAT1 (reviewed in Ref. 20). This is a so-called “tertiary active” process coupled indirectly via Na⁺-dicarboxylate cotransport to the Na⁺-K⁺ gradient, which is maintained by the Na⁺-K⁺-ATPase. Studies with isolated membrane vesicles have shown that excretion of PAH across the brush-border (apical) membrane into the urine include potential-driven facilitated transport as well as an anion exchange mechanism (13, 14). Neither system has yet been identified at the molecular level, whereas the apical organic anion transporters Oatp1 and Oat-1k do not appear to transport PAH (2, 6, 11, 15).

Multidrug resistance protein 2 (MRP2) is an ATP-dependent organic anion transporter and has been cloned from human, rat, and rabbit (7, 20). In the kidney, MRP2 has been localized to the apical (brush-border) membrane of cells of the proximal tubule (16, 17). Substrates of rat Mrp2 include conjugated organic anions, such as leukotriene C₄ (LTC₄), estradiol-17β-O-glucuronide (E₂17βG), and bilirubin-glucuronide, and unconjugated organic anions, such as bromosulphophthalein and folates (7, 18). In this study, we investigated whether MRP2 may also mediate ATP-dependent transport of PAH. Therefore, we determined uptake of [14C]PAH into membrane vesicles from Sf9 cells overexpressing recombinant rabbit Mrp2.

METHODS

Materials. [14C]PAH (53.1 mCi/mmol) and [6,7-3H]E₂17βG (55 Ci/mmol) were purchased from NEN Life Science Products (Hoofddorp, The Netherlands). Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim (Almere, The Netherlands). Fluorescein-methotrexate (FL-MTX), fura-acetoxymethyl ester (fura-AM), and lucifer yellow (LY) were obtained from Molecular Probes Europe (Leiden, The Netherlands). All other chemicals were obtained from Sigma (Zwijndrecht, The Netherlands).

Sf9-Mrp2 and control cells. Sf9 cells expressing rabbit Mrp2 (Sf9-Mrp2) were generated by infection of cells with a recombinant baculovirus encoding Mrp2 as described previously (21). Briefly, Sf9 cells (10⁶/ml) were grown as 100-ml suspension cultures and infected at a multiplicity of infection of one to five with Mrp2-encoding baculovirus. For control experiments, Sf9 cells infected with a baculovirus encoding the β-subunit of rat H⁺-K⁺-ATPase (Sf9-mock) were used.

Transport studies. Membrane vesicles from Sf9-Mrp2 and control cells were isolated as described (21). Uptake of...
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[14C]PAH or [3H]E$_2$17βG into membrane vesicles was performed as described (21). Briefly, membrane vesicles were thawed for 1 min at 37°C and added to prewarmed TS buffer (10 mM Tris-HEPES/250 mM sucrose pH 7.4) supplemented with an ATP-regeneration mix (4 mM ATP, 10 mM MgCl$_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase) and a labeled compound in a final volume of 60 μl. The reaction mixture was incubated at 37°C and at indicated times samples were taken from the mixture, diluted in 1 ml ice-cold TS buffer, and filtered through NC45 filters (Schleicher & Schuell, Dassel, Germany) by using a filtration device (Millipore, Bedford, MA). Filters were washed twice with 5 ml ice-cold TS buffer and dissolved in liquid scintillation fluid to determine the radioactivity bound. In control experiments, ATP was omitted from the reaction mixtures. Net ATP-dependent transport was calculated by subtracting values in the absence of ATP from those in the presence of ATP. Measurements were corrected for the amount of ligand bound to the filters (usually <2% of total radioactivity).

Determination of kinetic parameters. ATP-dependent [14C]PAH uptake into membrane vesicles of Sf9-Mrp2 and Sf9-mock cells could be described as uptake via a Michaelis-Menten process in parallel with nonspecific uptake. The Mrp2-mediated component of uptake was defined as the difference between ATP-dependent uptake in membrane vesicles of Sf9-Mrp2 and Sf9-mock cells. Curve fitting was done by least squares nonlinear regression analysis by using GraphPad software (GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA).

Statistics. Statistical comparison between the effect of various inhibitors on transport into membrane vesicles from Sf9-Mrp2 and Sf9-mock cells was done by one-factor ANOVA for repeated measurements. Within-group differences for inhibitors or inhibitor concentrations were assessed with a post hoc paired t-test, only when the repeated measures ANOVA showed a significant inhibitor effect. A P value of <0.05 (2-sided) was considered to indicate statistical significance.

RESULTS

We investigated uptake of [14C]PAH into membrane vesicles prepared from Sf9 cells infected with a baculovirus containing a full-length rabbit mrp2 cDNA (Sf9-Mrp2). We have recently shown that these membrane vesicles contain functional Mrp2 as assessed by ATP-dependent uptake of the anionic conjugates [3H]LTC$_4$ and [3H]E$_2$17βG (21). In the presence of ATP, membrane vesicles from Sf9-Mrp2 cells exhibited time-dependent uptake of [14C]PAH at 50 μM with initial rates of 25 ± 4 pmol·mg$^{-1}$·min$^{-1}$ (Fig 1A). In the absence of ATP, uptake was independent of time (50 ± 9 pmol/mg) and probably reflects nonspecific membrane binding (Fig 1A). The nonmetabolizable ATP analog 5’-AMP did not stimulate uptake of [14C]PAH (not shown). Compared with membrane vesicles of Sf9-mock cells, which exhibited endogenous ATP-dependent [14C]PAH uptake, initial transport rates in membrane vesicles of Sf9-Mrp2 cells were increased twofold to 20 ± 1 pmol·mg$^{-1}$·min$^{-1}$ (Fig 1B). Shrinking of the membrane vesicles by increasing extravesicular osmolarities resulted in reduced ATP-dependent [14C]PAH transport, providing evidence for uptake into the vesicular space (Fig. 2). Binding of [14C]PAH to the membrane vesicles in the presence of ATP decreased by 5–10% compared with binding in the absence of ATP (not shown).

To assess the kinetic parameters for [14C]PAH transport, uptake into membrane vesicles was performed at various [14C]PAH concentrations. In the presence of ATP, initial [14C]PAH uptake rates in Sf9-Mrp2 membrane vesicles increased with increasing PAH concentrations (Fig 3A). When corrected for nonspecific binding, uptake rates in both Sf9-Mrp2 and Sf9-mock membrane vesicles saturated at increasing [14C]PAH concentrations (Fig 3B). Correction for the endogenous transporter revealed a Michaelis-Menten constant ($K_m$) of 1.9 ± 0.8 mM and a maximal velocity ($V_{max}$) of 187 ± 29 pmol·mg$^{-1}$·min$^{-1}$ for Mrp2-mediated ATP-dependent [14C]PAH transport (Fig. 3C).

To further characterize ATP-dependent [14C]PAH transport by both Mrp2 and the endogenous transporter, we investigated the effect of various organic anions (Fig. 4). For all inhibitors a significant difference ($P < 0.01$) between Sf9-Mrp2 and Sf9-mock was found, indicating a difference in inhibitor specificity between Mrp2 and the endogenous transporter. Relatively higher concentrations of both E$_2$17βG and LTC$_4$ were required to inhibit...
ATP-dependent \[^{14}\text{C}\]PAH uptake by the endogenous transporter compared with Mrp2, but only for LTC\(_4\) was a significant inhibitory effect found in Sf9-mock. FL-MTX had no effect on the endogenous PAH transporter but inhibited Mrp2-mediated ATP-dependent \[^{14}\text{C}\]PAH uptake.

\(\alpha\)-Naphthyl-\(\beta\)-D-glucuronide had a stimulatory effect on ATP-dependent \[^{14}\text{C}\]PAH uptake in Sf9-Mrp2 membrane vesicles, whereas no effect was seen on uptake into Sf9-mock membrane vesicles. We also compared the effect of organic anions on ATP-dependent uptake of both \[^{14}\text{C}\]PAH and \[^{3}\text{H}\]\(\text{E}_2\)17\(\beta\)G (Fig. 5). Again a significant difference \((P < 0.01)\) between Mrp2 and the endogenous transporter was found. Probenecid, fura-AM, and LY did not inhibit the endogenous ATP-dependent PAH transporter but inhibited Mrp2-mediated \[^{14}\text{C}\]PAH transport by \(\sim 55\), 97, and 95\%, respectively. A similar inhibitory profile for these compounds was found on Mrp2-mediated ATP-dependent \[^{3}\text{H}\]\(\text{E}_2\)17\(\beta\)G uptake.

DISCUSSION

The present study demonstrates that the classic model compound for renal organic anion secretion, PAH, is a substrate for Mrp2, which is expressed at the brush-border membrane of renal proximal tubules. By using membrane vesicles from Sf9 cells expressing rabbit Mrp2, we demonstrated that \[^{14}\text{C}\]PAH uptake is time, osmotic, and ATP dependent. Kinetic parameters obtained for Mrp2-mediated ATP-dependent \[^{14}\text{C}\]PAH transport were a \(K_m\) value of 1.9 ± 0.8 mM and a \(V_{\text{max}}\) value of 187 ± 29 pmol·mg\(^{-1}\)·min\(^{-1}\). Sf9 cells appear to exhibit an endogenous ATP-dependent PAH transporter with a similar low affinity for PAH \((K_m\) of 1.4 ± 0.3 mM) as found for Mrp2. The dependency on ATP suggests that this endogenous transporter presumably is an MRP-like transporter with a substrate specificity, but one significantly different from that for Mrp2.

Whereas higher concentrations of \(\text{E}_2\)17\(\beta\)G and LTC\(_4\) were needed to inhibit the endogenous transporter, other compounds such as probenecid, fura-AM, FL-MTX, and LY did not inhibit at concentrations where Mrp2-mediated transport was partially or completely inhibited. Finally, \(\alpha\)-naphthyl-\(\beta\)-D-glucuronide stimulated ATP-dependent PAH transport by Mrp2 but not by the endogenous transporter. A stimulatory effect of compounds on Mrp2-mediated transport as shown here for \(\alpha\)-naphthyl-\(\beta\)-D-glucuronide has also been observed.

Fig. 2. Osmolarity dependency of ATP-dependent \[^{14}\text{C}\]PAH uptake into membrane vesicles of Sf9-Mrp2 and Sf9-mock cells. Uptake of \[^{14}\text{C}\]PAH (50 \(\mu\)M) into membrane vesicles was determined for 5 min in the presence of sucrose concentrations ranging from 250 (isotonic condition) to 1,000 mM. ATP-dependent transport was plotted against the inverse sucrose concentration in the reaction mixture. Linear fitting of the obtained data was performed by using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA). Data are means ± SD from 1 experiment performed in triplicate.

Fig. 3. Concentration dependency of ATP-dependent \[^{14}\text{C}\]PAH uptake into membrane vesicles of Sf9-Mrp2 and Sf9-mock cells. A: membrane vesicles from Sf9-Mrp2 cells were incubated with various concentrations of \[^{14}\text{C}\]PAH for 10 min in the absence (○) or presence (△) of ATP. B: net ATP-dependent transport of \[^{14}\text{C}\]PAH into membrane vesicles of Sf9-mock (○) and Sf9-Mrp2 (△) cells. C: corrected Mrp2-specific ATP-dependent \[^{14}\text{C}\]PAH transport. Lines through the data points are the fitted lines according to Michaelis-Menten kinetics (GraphPad). Data are means ± SE from 3 experiments performed in triplicate.
for reduced glutathione (3, 19), the sulfate conjugates of E3040 and methylumbelliferone (5, 12), penicillin G, and sulfinpyrazone (1).

Little is known about the molecular identity of the pathways for organic anion transport at the brush-border membrane. Studies in brush-border membrane vesicles using PAH as a substrate have indicated the presence of at least two transport systems, i.e., an anion exchange mechanism and potential-driven facilitated diffusion (13, 14). ATP-dependent PAH uptake has never been demonstrated in these vesicles. This may be explained by the inaccessibility of the ATP-binding site because of the almost complete right-side-out orientation of brush-border membrane vesicles from proximal tubular cells (4). In intact killifish renal proximal tubules, an energy-dependent efflux pathway

![Fig. 4. Inhibitory effect of estradiol-17β-D-glucuronide (A), leukotriene C4 (B), α-naphthyl-β-D-glucuronide (C), and fluorescein-methotrexate (D) on ATP-dependent [14C]PAH transport into membrane vesicles. ATP-dependent uptake of [14C]PAH (1 mM) into membrane vesicles from Sf9-Mrp2 (filled bars) and Sf9-mock (hatched bars) cells was determined for 10 min in the absence (control) or presence of E2,17βG, LTC4, α-naphthyl-β-D-glucuronide, or FL-MTX at concentrations as indicated. Data are means ± SD from a typical experiment performed in triplicate. The P values in the graphs represent ANOVA for comparison between groups (Mrp2 vs. mock). *P < 0.05 compared with ATP-dependent [14C]PAH uptake in Sf9-Mrp2 membrane vesicles in the absence of inhibitors. ‡P < 0.05 compared with ATP-dependent [14C]PAH uptake in Sf9-mock membrane vesicles in the absence of inhibitors (post hoc paired t-test).](image1)

![Fig. 5. Inhibitory effect of probenecid, fura-acetoxymethyl ester (fura-AM), and lucifer yellow (LY) on ATP-dependent transport of [14C]PAH (A) and [3H]E2,17βG (B) by Mrp2. ATP-dependent uptake of [14C]PAH (1 mM) and [3H]E2,17βG (50 nM) into membrane vesicles from Sf9-Mrp2 (filled bars) and Sf9-mock (hatched bars) cells was determined for 10 and 1 min, respectively, in the absence (control) or the presence of 0.1 M probenecid, 10 μM fura-AM, or 10 μM LY. Data are means ± SD from a typical experiment performed in triplicate. The P values in the figure represent ANOVA for comparison between groups (Mrp2 vs. mock). *P < 0.05 compared with ATP-dependent uptake in Sf9-Mrp2 membrane vesicles in the absence of inhibitors (post hoc paired t-test).](image2)
has been characterized for the organic anions FL-MTX and LY (8, 9). Luminal efflux of FL-MTX and LY was inhibited by the Mrp2 substrates LTC4 and (2,4-dinitrophenyl)-S-glutathione in a concentration-dependent manner (8, 9). Furthermore, immunohistochemistry with an antibody directed against rabbit Mrp2 revealed staining of the brush-border membrane (10). This indicates that an ortholog of rabbit Mrp2 is involved in luminal excretion of FL-MTX and LY in killifish proximal tubules. This is further corroborated by our finding that FL-MTX and LY appeared to be good inhibitors of Mrp2-mediated ATP-dependent transport of both PAH and E$_{p}$17βG.

Previous kinetic studies with isolated renal membrane vesicles have shown that both efflux pathways have a low affinity (K$_m$ 5–7 mM) for PAH (13, 14). The present results indicate that Mrp2 may be a third low-affinity pathway mediating the ATP-dependent renal excretion of PAH. In a preliminary experiment, we found inhibition of PAH efflux from isolated rat renal proximal tubules by (2,4-dinitrophenyl)-S-glutathione after preloading with 1-chloro-2,4-dinitrobenzene (R. Masereeuw, unpublished results). At this point, however, it is too soon to conclude from this result that Mrp2 has a predominant role in the renal excretion of PAH, because we cannot exclude that renal excretion of PAH involves multiple transport pathways. In this respect, efflux of FL-MTX from killifish proximal tubules is marginally inhibited by PAH, whereas efflux of LY is almost completely inhibited by PAH but only partly by LTC4 (8, 9). The involvement of multiple luminal transporters with overlapping substrate specificity makes it difficult to distinguish between these transporters just by using inhibitors. Therefore, more knowledge on the identity of luminal organic anion transporters and their subsequent knockout in mice is necessary. Whether Mrp2 contributes significantly to urinary PAH excretion will depend on its density at the brush-border membrane compared with other systems.

**NOTE ADDED IN PROOF**

While this study was under revision, Leier et al. (Kidney Int 57: 1636–1642, 2000) reported ATP-dependent PAH transport in membrane vesicles from HEK-293 cells expressing human Mrp2.

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**REFERENCES**