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ATP-dependent *para*-aminohippurate transport by apical multidrug resistance protein MRP2

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ATP-dependent *para*-aminohippurate transport by the apical multidrug resistance protein MRP2.

Background. *Para*-aminohippurate (PAH), a widely used model substrate for organic anion transport in proximal tubule epithelia, was investigated as a substrate for the apical multidrug resistance protein MRP2 (symbol ABCC2). This ATP-dependent export pump for anionic conjugates and additional amphiphilic anions was cloned recently and localized to the apical membrane of proximal tubules in human and rat kidney.

Methods. Membrane vesicles from HEK-MRP2 cells containing recombinant human MRP2 and from control vector-transfected HEK-Co cells were incubated with various concentrations of [^{3}H]PAH, and the net ATP-dependent transport into inside-out vesicles was determined. Comparative studies were performed with membrane vesicles containing recombinant human MRP1.

Results. Transport rates at 10 μmol/L PAH were 21.9 ± 1.9 and 1.6 ± 0.4 pmol × mg protein^{-1} × min^{-1} (means ± SEM, $N = 10$) with membrane vesicles from HEK-MRP2 and HEK-Co cells, respectively. The K_m value for PAH was 880 μmol/L. The high-affinity substrate leukotriene C_4 and the inhibitor of MRP-mediated transport, MK571, inhibited MRP2-mediated transport of PAH (100 nmol/L) with IC_{50} values of 3.3 and 4.0 μmol/L, respectively. The nephrotoxic mycotoxin ochratoxin A inhibited MRP2-mediated PAH transport with an IC_{50} value of 58 μmol/L. Ochratoxin A was itself a substrate for MRP2.

Conclusions. PAH is a good substrate for the ATP-dependent export pump MRP2. The localization and function of MRP2 indicate that this unidirectional transport protein contributes to the secretion of PAH and other amphiphilic anions into the lumen of kidney proximal tubules.

In the kidney, active transport predominates in the proximal tubules, and separate transport systems exist for the secretion of organic anions and cations into the tubular lumen [1–3]. Uptake across the basolateral membrane is followed by active transport across the apical (or luminal) membrane. Both processes are characterized by a high transport capacity and by a broad substrate specificity [1–3]. Whereas the uptake of organic anions has been defined relatively well, the mechanisms responsible for transport of compounds through the tubular cells and the secretion into the tubular lumen are less well understood. Among the secreted amphiphilic organic anions are drugs such as penicillins, cephalosporins, and diuretics, conjugated toxic agents, and the prototypic anion *para*-aminohippurate (PAH) [1, 3]. PAH secretion also involves a two-step process, starting with its uptake across the basolateral membrane of proximal tubule cells. In all species studied so far, extracellular PAH is exchanged for intracellular 2-oxoglutarate by the basolateral PAH/2-oxoglutarate antiporter cloned recently [4–8]. For the second step, the exit of PAH across the apical membrane into the tubular lumen, several different mechanisms have been discussed on the basis of studies with isolated renal brush border membrane vesicles, suggesting an antiporter for PAH secretion [9, 10]. Fluorescence imaging experiments characterized the transport of amphiphilic anions from tubular cells to the tubular lumen as a carrier-mediated uphill process [11, 12]. Cross-inhibition experiments suggested that in moving from cell to tubular lumen fluorescein, carboxyfluorescein diacetate, probenecid, PAH, and bimane conjugates all compete for a common transporter [11, 12].

Recently, MRP2, the multidrug resistance protein isoform characterized by its apical localization in polarized cells such as hepatocytes [13–16], has been identified in rat [17] and human [18] kidney, and localized to the apical membrane of proximal tubule epithelia. In earlier investigations, MRP2 was designated as the canalicular multidrug resistance protein [14] or as the canalicular multispecific organic anion transporter of hepatocytes [15]. MRP2 and the human multidrug resistance protein MRP1 [19] are presently the best characterized members of this family of adenosine 5′-triphosphate (ATP)-dependent exporter proteins. Human MRP1 is sorted to the basolateral membrane of transfected polarized cells [20] and is detected in many different cell types [21]. MRP2 and MRP1 share a similar and broad substrate specificity,

Key words: adenosine 5′-triphosphate-dependent transport, conjugate export pump, luminal transport, PAH, multiple drug resistance protein.

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which comprises the glutathione *S*-conjugate leukotriene C_4 (LTC_4) and many endogenous and xenobiotic lipophilic compounds conjugated to glutathione, glucuronate, or sulfate [22–28].

Of special interest for the overall handling of xenobiotics by the kidney in the intact organism is the interaction of the different transporters during secretion and reabsorption. The resulting intracellular concentrations of the xenobiotics play a pivotal role for their potential nephrotoxicity. Contamination of cereals and grains with *Aspergillus* and *Penicillium* fungi may lead to formation of the mycotoxin ochratoxin A (OTA) [29], which has been associated with the induction of Balkan nephropathy in humans and porcine nephropathy, as well as carcinogenesis [reviewed in 30]. The proximal tubule of the kidney is a primary site targeted in OTA-induced nephrotoxicity, and OTA was shown to interact with PAH and organic anion transporters both in the basolateral and the brush border membrane [31]. Moreover, acute OTA exposure impairs postproximal nephron function [30].

For the apical secretion of amphiphilic anions, including PAH and OTA, into the lumen of proximal tubules, MRP2 is a suitable candidate transporter because of its localization in the luminal membrane [17, 18] and because of its broad substrate specificity [26–28]. However, direct information on the role of MRP2 in the renal excretion of these xenobiotics has been lacking. Therefore, we used in the present study isolated membrane vesicles from HEK cells expressing recombinant human MRP2 for direct measurements of ATP-dependent transport of PAH and OTA. For comparison, transport mediated by the basolateral transporter MRP1 was determined. Potent inhibitors and substrates of ATP-dependent MRP-mediated transport, such as the anionic quinoline derivative MK571, cyclosporine A (CsA), and LTC_4 [14, 22, 23], served to characterize this transport process in further detail. Our studies demonstrate that MRP2 can function as a luminal PAH transporter in kidney proximal tubules and thus mediate, at least in part, the step in PAH transport identified earlier on the basis of functional studies [32].

METHODS

Materials

Para-amino[2-^{3}H-glycyl]hippurate (0.18 or 1.48 TB/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). PAH, CsA, and OTA were from Sigma Chemical Co. (St. Louis, MO, USA). MK571 (3-[{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]-thio)-propanoic acid was from Cayman Chemical (Ann Arbor, MI, USA). LTC_4 was purchased from Reatec GmbH (Weiterstadt, Germany). NICK-spin columns and Sephadex G50 fine were from Pharmacia Biotech (Freiburg, Germany). G418 (Geneticin®) was from Life Technologies (Eggenstein, Germany). Culture media and supplements were from Sigma-Aldrich Chemie (Deisenhofen, Germany).

Cells

Human embryonic kidney (HEK293) cells permanently expressing high levels of MRP2 (HEK-MRP2) were generated in our laboratory using the cDNA encoding human MRP2, as defined by the *EMBL/GenBank* accession X96395 [28]. Control HEK293 cells were transfected with the parental pcDNA3.1 vector (HEK-Co). All HEK cells were cultured in minimum essential medium (MEM) containing 10% fetal calf serum (FCS) supplemented with l-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL, 0.17 mmol/L). All cell lines were kept in a humidified incubator (5% CO_2, 37°C). HeLa T5 cells transfected with a vector containing the MRP1-coding sequence and control HeLa C1 cells transfected with the pRc/CMV vector [33] were kindly provided by Drs. R.G. Deeley and S.P.C. Cole (Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada). HeLa cell lines were grown in RPMI medium with 10% FCS under the selection pressure of G418 (Geneticin®).

Membrane vesicle preparation

Plasma membrane vesicles from HEK-MRP2, HEK-Co, HeLa T5, and HeLa C1 cells were prepared from hypotonically lyzed cells as described previously [22, 23, 26, 28]. In the case of HEK cell lines, butyrate (final concentration 5 or 10 mmol/L) was added to the culture medium 24 hours before membrane preparation to enhance MRP2 expression [28]. Membrane vesicles were frozen and stored in liquid nitrogen until use.

Vesicle transport measurements

Adenosine 5′-triphosphate-dependent transport of [^{3}H]PAH or of the fluorescent OTA was measured by centrifugation of the vesicles through a gel matrix using NICK-spin columns [26]. Membrane vesicles (20 μg protein) were incubated in the presence of 4 mmol/L ATP, 10 mmol/L $MgCl_2$, 10 mmol/L creatine phosphate, 100 μg/mL creatine kinase, and the substrate, in an incubation buffer containing 250 mmol/L sucrose and 10 mmol/L Tris/HCl, pH 7.4. The final incubation volume was 55 μL. The substrate and inhibitor concentrations are given in the respective figure legends. NICK-spin columns (0.17 g Sephadex G-50 per 3.3 mL) were prepared by rinsing with 250 mmol/L sucrose and 10 mmol/L Tris/HCl (pH 7.4) and centrifuged at 400 × *g* for four minutes at 4°C immediately before use. Aliquots (15 or 20 μL) of the incubations were taken at the indicated time points and immediately loaded onto Sephadex G-50 columns. The columns were rinsed with 180 μL of incubation buffer and centrifuged at 400 × *g* for four minutes at

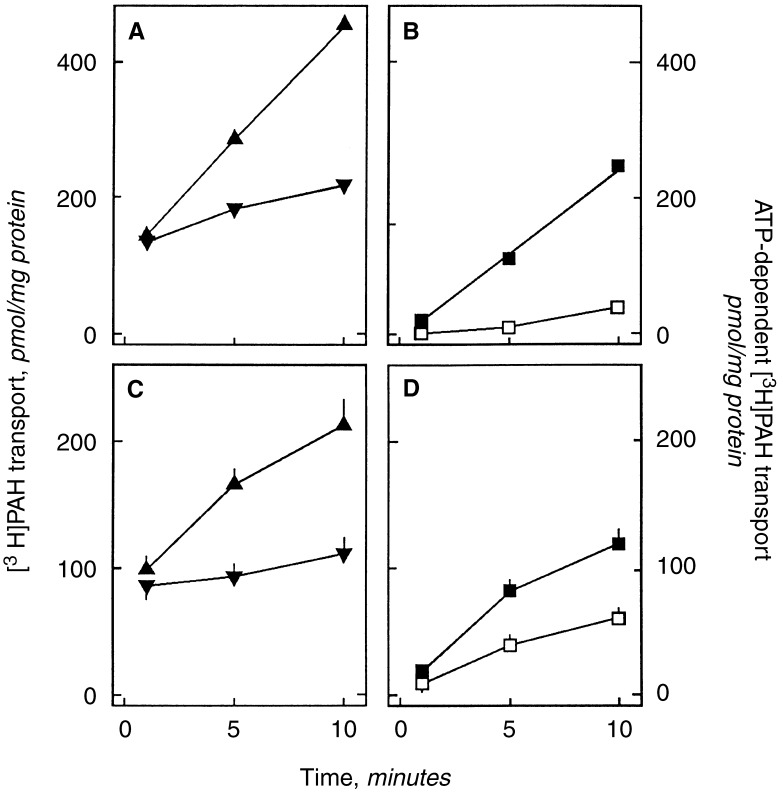

Fig. 1. Adenosine 5′-triphosphate (ATP)-dependent transport of [^{3}H] *para*-aminohippurate (PAH) mediated by the multidrug resistance proteins MRP2 and MRP1. Membrane vesicles (20 μg protein) from *MRP2*-transfected HEK-MRP2 cells and control vector-transfected HEK-Co cells (*A* and *B*), as well as membrane vesicles from *MRP1*-transfected HeLaT5 cells and control vector-transfected HeLaC1 cells (*C* and *D*), were incubated with 10 μmol/L [^{3}H]PAH in the presence of 4 mmol/L ATP (▲). In the control incubations, ATP was replaced by 4 mmol/L AMP-PCP (▼). The net ATP-dependent transport was calculated by subtracting values obtained in the presence of AMP-PCP from those obtained in the presence of ATP (■ in B and D). Control vector-transfected cell membrane vesicles exhibit a low rate of ATP-dependent transport (□ in B and D). Mean values ± SEM (N = 10) from two separate transport experiments with five separate transport incubations each are shown.

4°C. The effluents of the [^{3}H]PAH transport incubations were collected and assayed for vesicle-associated radioactivity. In control experiments, ATP was replaced by an equal concentration of the nonhydrolyzable ATP analogue AMP-PCP. Rates of net ATP-dependent transport were calculated by subtracting the blank values obtained in the presence of AMP-PCP from those measured in the presence of ATP. For determination of kinetic constants, ATP-dependent [^{3}H]PAH transport was measured in the concentration range from 50 to 1600 μmol/L.

The effluents of the incubations with the fluorescent OTA were collected into vials containing 20 μL of solubilization buffer (1% Triton X-100 in Tris/HCl, pH 7.4; final concentration 0.1% Triton X-100) to release the intravesicularly trapped fluorescent substrate by the detergent [34]. Subsequently, the samples were diluted with 600 μL 0.1% Triton X-100, 225 mmol/L sucrose, and 20 mmol/L Tris/HCl (pH 7.4). An aliquot of 800 μL of each sample was measured fluorometrically (RF-510 fluorescence spectrophotometer; Shimadzu, Duisburg, Germany) at an excitation wavelength of 375 nm and an emission wavelength of 440 nm [35] at a gain setting of 50 and an averaging time of one second. A calibration curve (0 to 0.5 nmol/L OTA) was prepared in triplicate to calculate the amount of OTA transported into the HEK-MRP2 membrane vesicles.

RESULTS

ATP-dependent PAH transport mediated by MRP2 and MRP1

To identify PAH as a substrate for the apical multidrug resistance protein, MRP2, membrane vesicles from cells expressing recombinant MRP2 were incubated with 10 μmol/L [^{3}H]PAH in the presence of ATP or, for control measurements, in the presence of the nonhydrolyzable ATP analogue AMP-PCP (Fig. 1A). For comparison, corresponding assays were performed with membrane vesicles from HeLa T5 cells expressing recombinant MRP1 (Fig. 1C). The ATP-dependent transport rates (Fig. 1 B, D) demonstrate that [^{3}H]PAH is transported by MRP2 (Fig. 1C), as well as by MRP1 (Fig. 1D). In addition, transport of 10 μmol/L [^{3}H]PAH was measured under the same conditions with membrane vesicles from the respective vector-transfected control cells, which express a low level of endogenous MRP isoforms [28, 33]. The respective rates for ATP transport were 21.9 ± 1.9, 1.6 ± 0.4, 17.4 ± 1.9, and 7.9 ± 1.8 pmol × mg protein^{-1} × min^{-1} (means ± SEM, N = 10) for HEK-MRP2, HEK-Co, HeLa T5, and HeLa C1 membrane vesicles, respectively. Similar transport assays were performed at 10 μmol/L [^{3}H]PAH with membrane vesicle suspensions from MRP2 (Fig. 2) as well as from MRP1 transfectants in order to study the effect of medium osmolarity. Transport rates

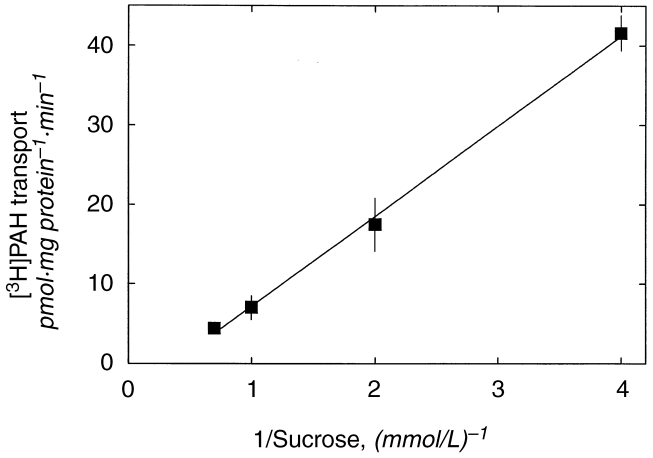

Fig. 2. Osmolarity dependence of ATP-dependent MRP2-mediated [^{3}H]PAH transport. Membrane vesicles (20 μg protein) from *MRP2*-transfected HEK-MRP2 cells were incubated with 10 μmol/L [^{3}H]PAH in the presence of 4 mmol/L ATP, or in control incubations with 4 mmol/L AMP-PCP, in suspensions containing sucrose concentrations ranging from 250 mmol/L (isotonic condition) to 1500 mmol/L. Net ATP-dependent transport, calculated as described in the legend to Figure 1, is plotted against the reciprocal sucrose concentration in the transport incubation. Mean values ± SEM (N = 8) from two separate experiments with four separate transport incubations each are shown.

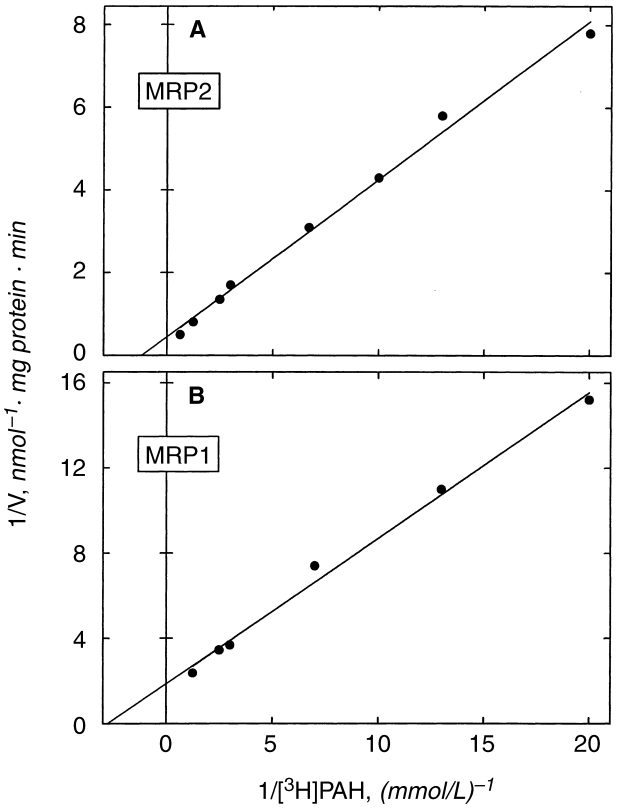

Fig. 3. Determination of the kinetic constants for MRP2- and MRP1-mediated [^{3}H]PAH transport. Membrane vesicles from HEK-MRP2 and HeLa T5 cells expressing recombinant MRP2 and MRP1, respectively, were incubated with PAH in the concentration range from 50 to 1600 μmol/L. The net ATP-dependent transport rates were calculated, and the kinetic constants were determined according to Lineweaver and Burk. (*A*) Mean values from four separate experiments, each performed with duplicate determinations. (*B*) Mean values from two separate experiments, each with duplicate determinations.

obtained with both vesicle suspensions decreased with increasing concentrations of sucrose, indicating transport into an osmotically sensitive space and not binding to the vesicle surface.

Kinetic constants for MRP2- and MRP1-mediated ATP-dependent PAH transport

For determination of K_m and V_{max} values for PAH transport mediated by MRP2 and MRP1, transport was measured in the concentration range of 50 to 1600 μmol/L (Fig. 3). The K_m value for MRP2 was 880 μmol/L and the one for MRP1 was 372 μmol/L, and the corresponding V_{max} values were 2.3 and 0.54 nmol × mg protein^{-1} × min^{-1} for MRP2 and MRP1, respectively.

Inhibition of MRP-mediated ATP-dependent PAH transport

Membrane vesicles from HEK-MRP2 and HeLa T5 cells were incubated with 100 nmol/L [^{3}H]PAH in the presence of the high-affinity MRP substrate LTC_4, the MRP inhibitor MK571, and CsA at the concentrations indicated in Figure 4. LTC_4 and MK571 inhibited the MRP2- and the MRP1-mediated [^{3}H]PAH transport markedly, with IC_{50} values between 3.3 and 4.9 μmol/L (Fig. 4). CsA inhibited MRP1-mediated transport with an IC_{50} value of 3.3 μmol/L, whereas the IC_{50} value for MRP2 was above 10 μmol/L, in a concentration range in which CsA solubility becomes limiting.

Effect of OTA on MRP-mediated PAH transport

The nephrotoxic mycotoxin OTA was tested as a possible inhibitor of ATP-dependent [^{3}H]PAH transport. Membrane vesicles from HEK-MRP2 and HeLa T5 cells were incubated with 100 nmol/L [^{3}H]PAH in the presence of OTA at concentrations between 10 and 100 μmol/L. The IC_{50} values were about 58 μmol/L for MRP2 and 53 μmol/L for MRP1, as shown in Figure 5.

Identification of OTA as a substrate for MRP2-mediated transport

Membrane vesicles from HEK-MRP2 were incubated with OTA to test whether this fluorescent compound is a substrate for MRP2. As shown in Figure 6, OTA was transported by MRP2. The net ATP-dependent transport for OTA, at a concentration of 200 μmol/L, was 1.2 ± 0.1 nmol × mg protein^{-1} × min^{-1} (mean ± SD, N = 4).

DISCUSSION

Our results indicate that MRP2, the multidrug resistance protein isoform localized to the apical membrane

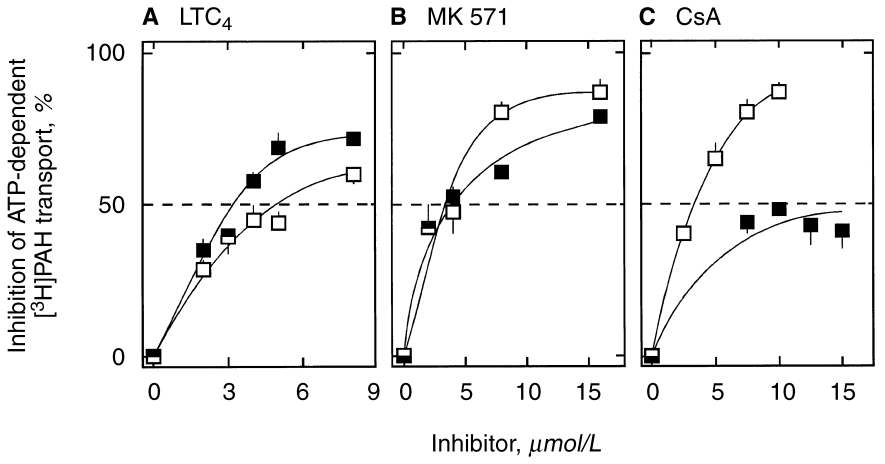

Fig. 4. Inhibition of ATP-dependent [^{3}H]PAH transport, mediated by recombinant MRP2 (■) or MRP1 (□), by (*A*) LTC_4, (*B*) MK 571, and (*C*) CsA. Transport of 100 nmol/L [^{3}H]PAH was measured in membrane vesicles from HEK-MRP2 and HeLa T5 cells, expressing recombinant MRP2 and MRP1, respectively, in the presence or absence of inhibitors, as indicated. The net ATP-dependent transport and percentage of inhibition were calculated for each inhibitor concentration in comparison to control incubations without inhibitor. Transport rates for MRP2- and MRP1-mediated transport in the absence of inhibitor were 0.68 ± 0.03 pmol $\times$ mg protein$^{-1} \times$ min^{-1} and 0.40 ± 0.02 pmol $\times$ mg protein$^{-1} \times$ min^{-1}, respectively. In A, the IC_{50} for LTC_4 is 3.3 μmol/L for MRP2 and 4.9 μmol/L for MRP1; in B, the IC_{50} for MK571 is 4.0 μmol/L for MRP2 and 3.3 μmol/L for MRP1; in C, the IC_{50} for CsA is >10.0 μmol/L for MRP2 and 3.3 μmol/L for MRP1. Mean values ± SEM from eight different experiments, each with five duplicate determinations, are shown. Each inhibitor concentration was tested in two separate incubations, with five duplicate determinations each.

of polarized cells, is part of the PAH transport systems in proximal tubule epithelia of the kidney. Measurements of ATP-dependent transport into membrane vesicles from HEK cells expressing recombinant MRP2 [28] demonstrate that PAH is a good substrate for this apical export pump (Figs. 1 and 3). The V_{max}/K_m ratio for PAH as a substrate for MRP2 was 2.6 μL $\times$ mg$^{-1} \times$ min^{-1} (Fig. 3), as compared with 20 and 351 μL $\times$ mg$^{-1} \times$ min^{-1} for the prototypic substrates 17β-glucuronosyl estradiol and LTC_4, respectively [28]. In addition, PAH was transported by recombinant human MRP1 (Figs. 1 and 3). This is in line with the observations indicating that MRP2 and MRP1 have a similar substrate specificity [26, 28]. PAH transport was inhibited by similar concentrations of LTC_4 and of the LTD_4 analogue MK571, as well as by CsA, which is a known inhibitor for several ATP-dependent export pumps [36] (Fig. 4). CsA and LTC_4 were also shown to inhibit organic anion secretion into the lumen of intact kidney proximal tubules, suggesting the presence of an MRP2-like apical transporter [12, 37]. Although additional MRP isoforms (for example, MRP3-6) have been identified recently [38], MRP2 is the only isoform detected in the apical membrane domain of polarized cells [27, 28, 39]. No information is currently available on the localization of endogenous MRP1 in kidney, although transfection studies have indicated that recombinant MRP1 is sorted to the lateral membrane of porcine kidney cells [20]. Another basolateral isoform of the MRP family, MRP3, is expressed in human kidney only at a very low level [39]. Thus, there is no evidence at present suggesting a functionally important role of MRP isoforms other than MRP2 in proximal tubules

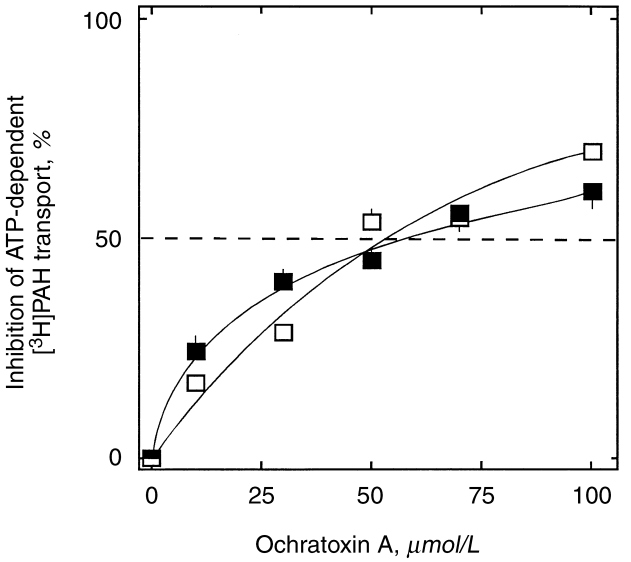

Fig. 5. Inhibition of ATP-dependent MRP2- and MRP1-mediated PAH transport by ochratoxin A (OTA). Transport of 100 nmol/L [^{3}H]PAH was measured in membrane vesicles containing recombinant MRP2 (■) or MRP1 (□), in the presence of the OTA concentrations indicated. The net ATP-dependent transport and percentage of inhibition were calculated for each inhibitor concentration in comparison to control incubations without inhibitor. Transport rates for MRP2- and MRP1-mediated transport in the absence of OTA are given in the legend to Figure 4. The IC_{50} is 53 μmol/L for MRP1 and 58 μmol/L for MRP1. Each inhibitor concentration was tested in two separate incubations with five duplicate determinations each.

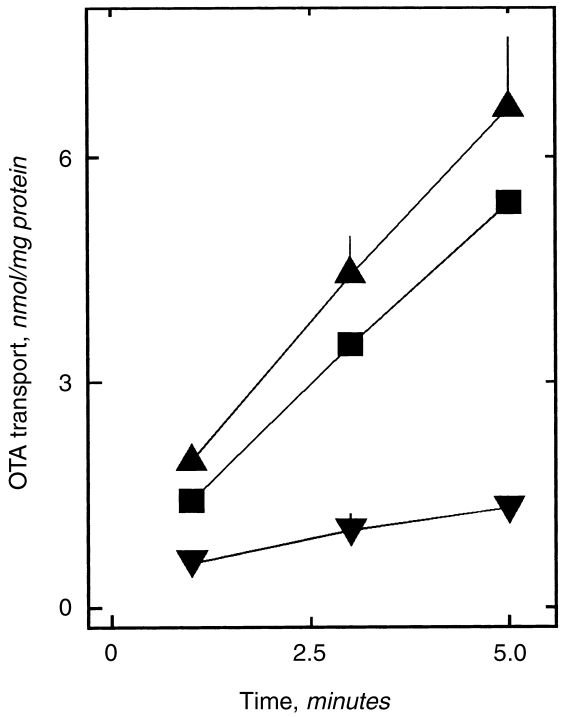

Fig. 6. ATP-dependent transport of ochratoxin A (OTA) mediated by the apical multidrug resistance protein MRP2. Membrane vesicles (20 μg protein) from *MRP2*-expressing HEK-MRP2 were incubated with 200 μmol/L OTA in the presence of 4 mmol/L ATP (▲). Control incubations were performed in the presence of 4 mmol/L AMP-PCP (▼). Vesicle-associated OTA fluorescence was determined as described in the **Methods** section, and the net ATP-dependent transport was calculated by subtracting the values obtained in the presence of AMP-PCP from those obtained in the presence of ATP (■). Mean values ± SD from four separate incubations are shown.

of the kidney. In analogy to the role of MRP2 in the hepatobiliary elimination of amphiphilic organic anions [16], we conclude that it is MRP2 that decisively contributes to the secretion of these substances by proximal tubule epithelia into the tubular lumen. If basolaterally localized MRP isoforms are expressed in kidney proximal tubules at a low level, they should not affect the overall direction of secretion of PAH by proximal tubules.

The inhibition of MRP2- and MRP1-mediated PAH transport by the mycotoxin OTA (Fig. 5) is in line with previous investigations showing that OTA interferes with organic anion transport in brush border membrane vesicles [31], as well as with organic anion efflux across the apical membrane in proximal tubule-derived opossum kidney cells [40]. Vice versa, a reduction of OTA secretion in primary cultures of rabbit proximal tubules by PAH has been shown [35].

MRP2 is the first cloned ATP-dependent export pump for amphiphilic organic anions identified in human and rat kidney, and is localized to the apical membrane of proximal tubule epithelia [17, 18]. Further to MDR1 P-glycoprotein [41], MRP2 is the second ATP-binding cassette transporter localized to the proximal tubule apical membrane. MDR1 P-glycoprotein markedly differs in its function and amino acid sequence from MRP1 and MRP2 [16, 19]. MDR1 P-glycoprotein functions in the ATP-dependent export of a broad spectrum of lipophilic, mostly cationic, substances [41, 42], whereas MRP2 and MRP1 mediate the transport of amphiphilic anions such as leukotriene LTC_4 and several other endogenously formed glutathione, glucuronate, and sulfate conjugates [22–28]. Moreover, MRP1 and MRP2 also transport certain unconjugated compounds, such as the amphiphilic penta-anion Fluo-3 [34] and, as shown in this study, the amphiphilic anions PAH and OTA (Figs. 1 and 6).

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APPENDIX

Abbreviations used in this article are: CsA, cyclosporin A; HEK293, human embryonic kidney cell line; HEK-Co, HEK cells transfected with the parental pcDNA3.1 vector; HEK-MRP2, HEK cells stably transfected with *cDNA* encoding human *MRP2*; HeLa C1, HeLa cells transfected with the pRc/CMV vector; HeLa T5 cells, HeLa cells transfected with a vector containing the MRP1-coding sequence; LTC_4, leukotriene C_4; MEM, minimum essential medium; MK571 (3-[[3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl]-{(3-dimethylamino-3-oxopropyl)-thio}-methyl]-thio)-propanoic acid; MRP1, multiple drug resistance protein 1 (also multidrug resistance-associated protein; symbol ABCC1); MRP2, apical isoform of MRP family (also cMRP, for canalicular multidrug resistance protein, or cMOAT for canalicular multispecific organic anion transporter; symbol ABCC2); OTA, ochratoxin A; PAH, *para*-aminohippurate.

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