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Cloning and Functional Characterization of a New Multispecific Organic Anion Transporter, OAT-K2, in Rat Kidney

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ABSTRACT

We have isolated a cDNA coding a new organic anion transporter, OAT-K2, expressed specifically in rat kidney. The OAT-K2 cDNA had an open reading frame encoding a 498-amino acid protein (calculated molecular mass of 55 kDa) that shows 91% identity with the rat kidney-specific organic anion transporter, OAT-K1. Reverse transcription-coupled polymerase chain reaction analyses revealed that the OAT-K2 mRNA was expressed predominantly in the proximal convoluted tubules, proximal straight tubules, and cortical collecting ducts. When expressed in *Xenopus* oocytes, OAT-K2 stimulated the uptake of hydrophobic organic anions, such as taurocholate, methotrexate, folate, and prostaglandin E_2, although its homolog OAT-K1 transported methotrexate and folate, but not taurocholate and prostaglandin E_2. In MDCK cells stably transfected with the OAT-K1 and OAT-K2 cDNAs, each transporter was localized functionally to the apical membranes and showed transport activity similar to that in the oocyte. Moreover, the efflux of preloaded taurocholate was also enhanced across the apical membrane in OAT-K2 transfectant. The taurocholate transport by OAT-K2-expressing cells showed saturability (K_m = 10.3 μM). Several organic anions, bile acids, cardiac glycosides, and steroids had potent inhibitory effects on the OAT-K2-mediated taurocholate transport in the transfectant. These findings suggest that the OAT-K2 participates in epithelial transport of hydrophobic anionic compounds in the kidney.

A diverse array of organic anions including endogenous substances, xenobiotics, and their metabolites are disposed from the body. The kidney is critical in the elimination of anionic drugs. The net drug excretion into urine is defined basically by three processes: glomerular filtration, tubular secretion, and reabsorption. The proximal tubular cells play a principal role in limiting or preventing toxicity by actively secreting anions from the circulation into the urine (Pritchard and Miller, 1993; Ullrich, 1997; Inui and Okuda, 1998). The organic anion secretion system is a complicated transport process recognizing a wide variety of substrates at the brush-border and basolateral membranes of the proximal tubule (Ullrich, 1997). Recently, a renal basolateral-type organic anion/dicarboxylate exchanger, OAT-1/ROAT1, has been cloned and characterized (Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997). OAT-1/ROAT1 is suggested to mediate the basolateral entry of various organic anions into the proximal tubular epithelial cells. In contrast, the brush-border-type transport system, which mediates the secretion of various types of organic anions from cell to lumen, was not characterized.

We recently isolated cDNA encoding a rat kidney-specific organic anion transporter, OAT-K1, mediating transport of methotrexate and folate but not *p*-aminohippurate and taurocholate in the kidney (Saito et al., 1996). OAT-K1 mRNA transcript and its product are expressed only in the kidney, especially in the brush-border membranes of the proximal straight tubules (Masuda et al., 1997b). We suggested that OAT-K1 transporter mediates facilitative translocation of methotrexate in the renal brush-border membranes. Because the renal organic anion transporters mediate secretion of various organic anions in the brush-border membranes, several transporters including OAT-K1-related proteins should be expressed to compose a multispecific organic anion secretion system peculiar to the kidney.

We report here the identification of a new organic anion transporter, OAT-K2, which was isolated from a rat kidney cDNA library. Functional analyses showed that the rat OAT-K2 functions as a multispecific organic anion transporter in the renal brush-border membranes.

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB012662.

ABBREVIATIONS: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; oatp, organic anion-transporting polypeptide; PMCA, plasma membrane calcium-pumping ATPases; PCR, polymerase chain reaction; RT-PCR, reverse transcription-coupled polymerase chain reaction.

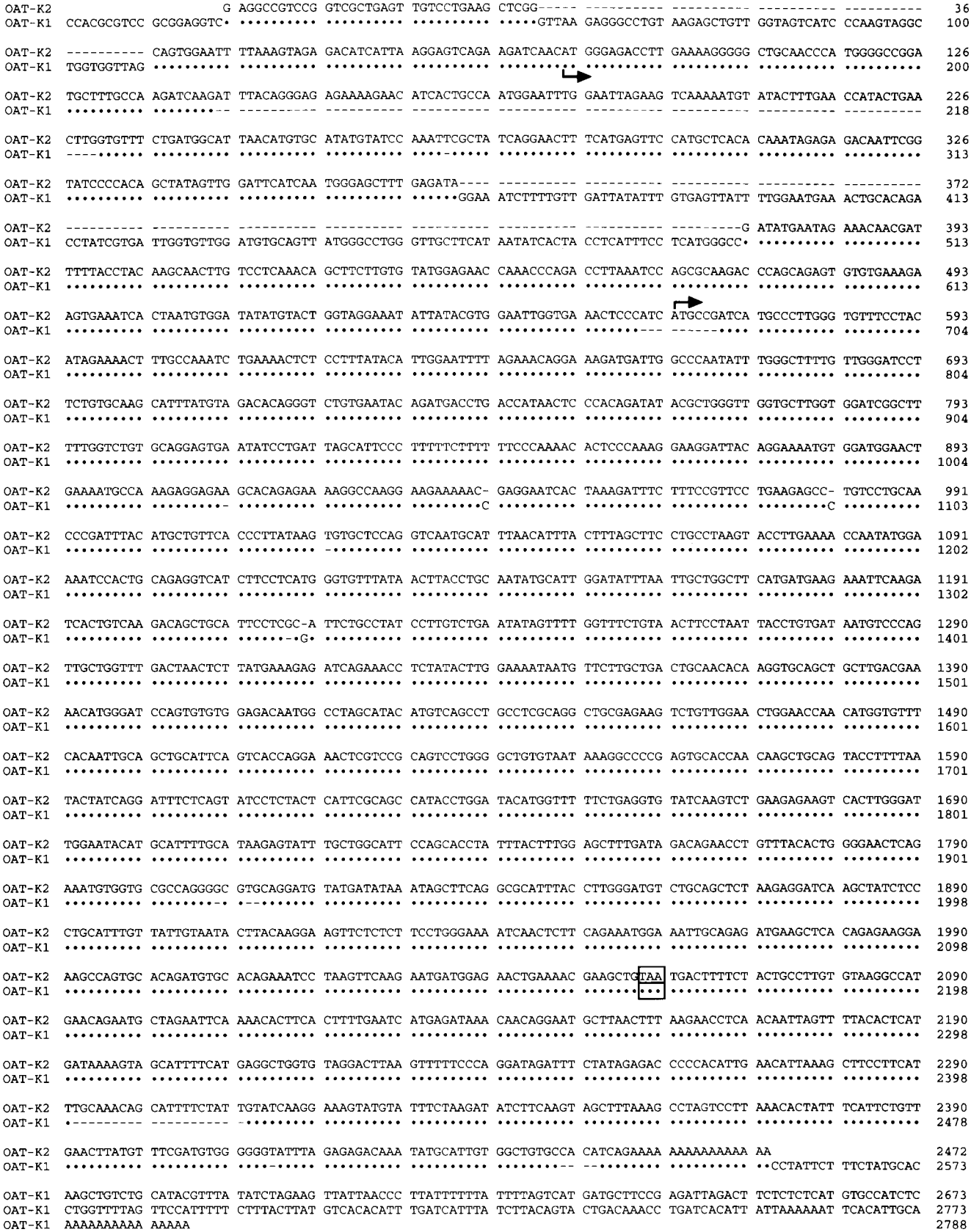

Fig. 1. Comparison of the nucleotide sequences between rat OAT-K1 and OAT-K2. Conserved nucleotides between two transporters are indicated by dots. The start codon of each transporter is indicated by an arrow. The stop codon of each transporter is boxed.

Experimental Procedures

Materials. [^{3}H]Taurocholate (128.39 GBq/mmol), [G-^{3}H]digoxin (592 GBq/mmol), [1,2,6,7-^{3}H-N]testosterone (3,222.7 GBq/mmol), and [2-^{14}C]indomethacin (825.1 MBq/mmol) were obtained from DuPont-New England Nuclear Research Products (Boston, MA). [^{3}H]Prostaglandin E_2 (6700 GBq/mmol) and [3′,5′7-^{3}H]methotrexate sodium salt (359 GBq/mmol) were from Amersham Int. (Buckinghamshire, UK). [3′,5′7,9-^{3}H]Folate was from Moravek Biochemical, Inc. (Brea, CA). Levofloxacin was supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Probenecid, 4,4′-diisothiocyano-2,2′-disulfonic stilbene, and taurochenodeoxycholate were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled methotrexate, unlabeled indomethacin, dexamethasone, and valproate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Unlabeled taurocholate, glycocholate, deoxycholate, taurodeoxycholate, glycochenodeoxycholate, ursodeoxycholate, sulfobromophthalein, *p*-aminohippurate, furosemide, benzylpenicillin, digoxin, prednisolone, spironolactone, testosterone, estriol, and estradiol were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used for the experiments were of the highest purity available.

Screening of the cDNA Library. The oligo(dT)-primed directional rat kidney cDNA library (Uchida et al., 1993), which was used for the cDNA isolation of rat OAT-K1, was screened by hybridization with a polymerase chain reaction (PCR) clone labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) as described (Saito et al., 1996). Rat OAT-K2, a positive clone, was isolated with a 2.5-kb insert and was subcloned into *Sal*I- and *Not*I-cut pSPORT1, and then sequenced on both strands with synthetic oligonucleotide primers.

Northern blot and reverse transcription-coupled PCR (RT-PCR) Analyses. After extraction of total RNA from several tissues of male Wistar rats (220–240 g), poly(A)$^+$ RNA was purified by oligo(dT)-cellulose (Collaborative Research Inc., Bedford, MA) affinity column chromatography, as described previously (Saito et al., 1996). For Northern blot analysis, 3 μg of poly(A)$^+$ RNA from rat tissues was resolved by electrophoresis in 1% agarose gels containing formaldehyde and transferred onto nylon membranes. After transfer, blots were hybridized at high stringency (50% formamide, 5× SSPE (20× SSPE; 3M NaCl, 0.2 M NaH_2PO_4, 0.02M EDTA; pH 7.4), 5× Denhardt's solution, 0.2% SDS, and 10 μg/ml salmon sperm DNA at 42°C) with a whole OAT-K2 cDNA labeled with [α-^{32}P]dCTP as described above. To ensure the presence of poly(A)$^+$ RNA in each lane, the same blot was subsequently probed with [α-^{32}P]dCTP-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (Tso et al., 1985). For RT-PCR analysis, 1 μg of poly(A)$^+$ RNA from tissues was reverse transcribed and amplified according to the following profile: 94°C for 1 min, 58°C for 1 min, 72°C for 2.5 min, 30 cycles, with either a set of primers specific for the nucleotide sequence of rat OAT-K2 [sense strand, 5′-GAACATCACTGCCAATGGAA-3′ (bases 163 ~ 182); antisense strand, 5′-ACACAAGGCAGTAGAAAAGT-3′ (bases 2064 ~ 2083)] or primers specific for the rat GAPDH [sense strand, 5′-CGGCCTCGTCTCATAGACAA-3′ (bases 10 ~ 29); antisense strand, 5′-TGGTCCAGGGGTTTCTTACT-3′ (bases 1028 ~ 1047)]. The rat OAT-K1 cRNA and rat OAT-K2 cRNA were reverse transcribed and amplified with each set of these primers as control templates.

RT-PCR with Microdissected Nephron Segments. Microdissection of nephron segments (five glomeruli and 2 mm of each dis-

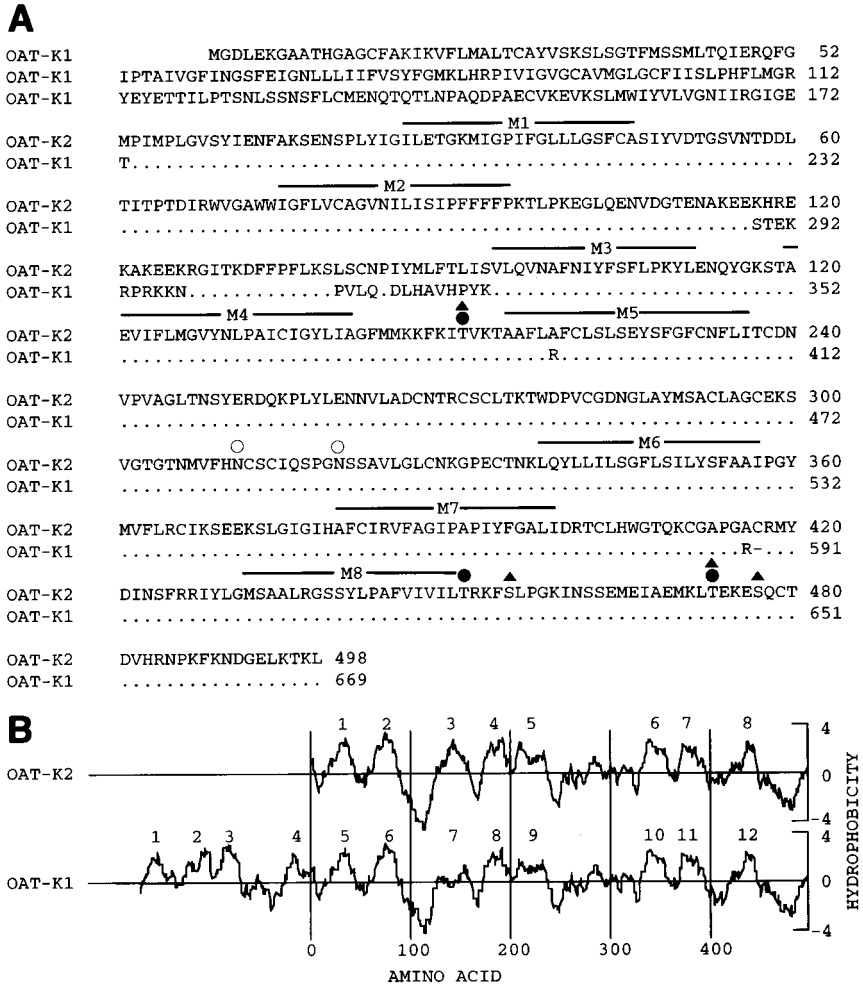

Fig. 2. Comparison of the deduced amino acid sequences (A) and hydropathy plots (B) between rat OAT-K1 and OAT-K2. A, conserved residues between two transporters are indicated by dots. Putative membrane-spanning domains are lined over the sequence with the number (M1–M8), and potential *N*-linked glycosylation sites (○), potential protein kinase A phosphorylation sites (▲), and protein kinase C phosphorylation sites (●) are indicated. B, Kyte and Doolittle (1987) hydropathy plots with a window of 13 amino acid residues. Numbers between plots indicate putative membrane-spanning regions.

sected tubule segment) and reverse transcription of mRNA were performed as described (Masuda et al., 1997b). A set of primers specific for the nucleotide sequence of rat OAT-K2 was used [sense strand, 5′-GAACATCACTGCCAATGGAA-3′ (bases 163 ~ 182); antisense strand, 5′-CTTATAAGGGTGAACAGCATG-3′ (bases 1002 ~ 1022)]. The PCR profile was the same as that described above. The expected size of PCR product from OAT-K2 was 860 bp. For Southern blot analysis, the blot was hybridized with a whole OAT-K2 cDNA labeled with [α-^{32}P]dCTP as described above.

Uptake Study in *Xenopus* Oocytes. After linearization of the constructed cDNA pSPORT1/OAT-K1 and pSPORT1/OAT-K2 by digestion with *Not*I, each capped cRNA was transcribed in vitro by use of T7 RNA polymerase (Stratagene, La Jolla, CA). Twenty nanograms of transcript was injected into isolated *Xenopus* oocytes, and uptake studies were performed as described (Saito et al., 1995).

Cell Culture and Transfection. The parental MDCK cells were cultured in complete culture medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc., Rockville, MD) with 10% fetal calf serum (Microbiological Associates, Bethesda, MD) in an atmosphere of 5% CO_2/95% air at 37°C. OAT-K2 cDNA was subcloned into the *Sal*I- and *Not*I-cut mammalian expression vector pBK-CMV (Stratagene) (Brewer, 1994). MDCK cells were transfected with pBK-CMV/OAT-K2 or pBK-CMV using the calcium phosphate coprecipitation technique, as described previously (Saito et al., 1996). After selection in 0.5 mg/ml G418 (Life Technologies, Inc.) for 8 to 10 days, single colonies were picked up with cloning cylinders for subsequent screening. G418-resistant clonal cells were analyzed by both RT-PCR and Northern blotting for the expression of rat OAT-K2 mRNA. For the transport experiments, cells were seeded in the complete medium on 35-mm-diameter culture dishes or microporous membrane filters inside a Transwell cell culture chamber (Costar, Cambridge, MA).

Uptake Study in MDCK Cells Stably Expressing OAT-K2. Cellular uptake of radioactive drugs was measured with monolayer cultures grown in 35-mm diameter dishes. The incubation medium for uptake experiments was Dulbecco's PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$; pH 7.4), containing 5 mM D-glucose (uptake buffer). In Na^+-free medium, the NaCl and Na_2HPO_4 of the uptake buffer were replaced with *N*-methyl-D-glucamine and K_2HPO_4, respectively. In Cl^--free medium, NaCl, KCl, $CaCl_2$, and $MgCl_2$ were replaced with sodium gluconate, potassium gluconate, calcium gluconate, and $MgSO_4$, respectively (Saito et al., 1992). In the transport studies, the total uptake was determined for radiolabeled drug alone. For directional uptake or efflux studies, uptake measurements were performed using Transwell chambers as described previously but with some modifications (Saito et al., 1992; Takano et al., 1994). At the end of the incubation, cells were washed once in the uptake buffer with 1% of BSA and three more times in ice-cold BSA-free uptake buffer. The protein content of the solubilized cells in 0.5 N NaOH solution was determined by the method of Bradford (1976), using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) with the bovine γ-globulin as a standard.

Statistical Analysis. Data were analyzed statistically using one-way ANOVA followed by Fisher's *t* test.

Results

cDNA Cloning of the Rat OAT-K2. With a PCR product of about 270 bp, which was homologous (~80% nucleotide identity) to the rat liver organic anion transporting polypeptide oatp1 cDNA, originating from the rat kidney cortex as a probe, a rat kidney λgt22A cDNA library was screened under high stringency. After repeated screening, we obtained a 2.5-kb cDNA clone designated as rat OAT-K2, which was distinct from the rat OAT-K1. Figure 1 shows the nucleotide sequence of OAT-K2 cDNA in comparison with that of OAT-K1. The OAT-K2 cDNA consists of 2472 bp with 415 bp of noncoding nucleotides at the 3′ end and with a poly(A)$^+$ tail. Based on the Kozak consensus sequence (1987), the initiation site was assigned to the ATG codon at position 564. Consequently, the open reading frame of the cloned OAT-K2 cDNA extends over 1494 nucleotides, coding for a 498-amino acid protein with a calculated molecular mass of 55 kDa. Figure 2A shows the deduced amino acid sequence of rat OAT-K2 and its alignment with its homolog OAT-K1. Rat OAT-K2 and OAT-K1 (Saito et al., 1996) showed an amino acid identity of 91%. OAT-K2 also showed amino acid identity of 65% with rat organic anion-transporting polypeptide (oatp)1 (Jacquemin et al., 1994), 62% with rat oatp2 (Noé et al., 1997), 63% with rat oatp3 (Abe et al., 1998), 53% with human OATP

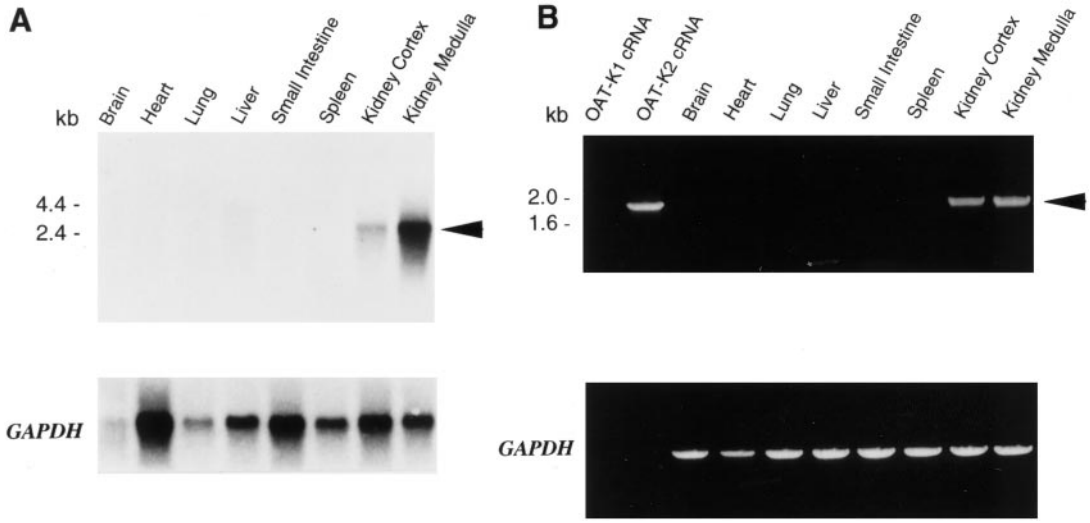

Fig. 3. Northern blot analysis (A) and detection by PCR amplification (B) of OAT-K2 mRNA in rat tissues. A, three micrograms of poly(A)$^+$ RNA from the indicated tissues was electrophoresed, blotted, and hybridized with the whole OAT-K2 cDNA (upper) or GAPDH cDNA (lower) as a probe at high stringency. B, one microgram of poly(A)$^+$ RNA from the indicated tissues, OAT-K1 cRNA (25 ng), and OAT-K2 cRNA (25 ng) were reverse transcribed and amplified using a set of primers specific for the OAT-K2 (upper) or for the GAPDH (lower) as described in the text. The PCR products were separated by electrophoresis through 1% agarose gels and stained with ethidium bromide.

(Kullak-Ublick et al., 1995), 31% with rat prostaglandin transporter (PGT) (Kanai et al., 1995), and 31% with human PGT (Lu et al., 1996). A Kyte-Doolittle (1982) hydropathy analysis suggested that rat OAT-K2 has eight putative membrane-spanning domains (Fig. 2B), thus indicating two potential *N*-linked glycosylation sites in the extracellular loop. There are four potential cAMP-dependent kinase phosphorylation sites at positions 211, 455, 473, and 477, and three potential protein kinase C phosphorylation sites at positions 211, 451, and 473 (Kennelly and Krebs, 1991).

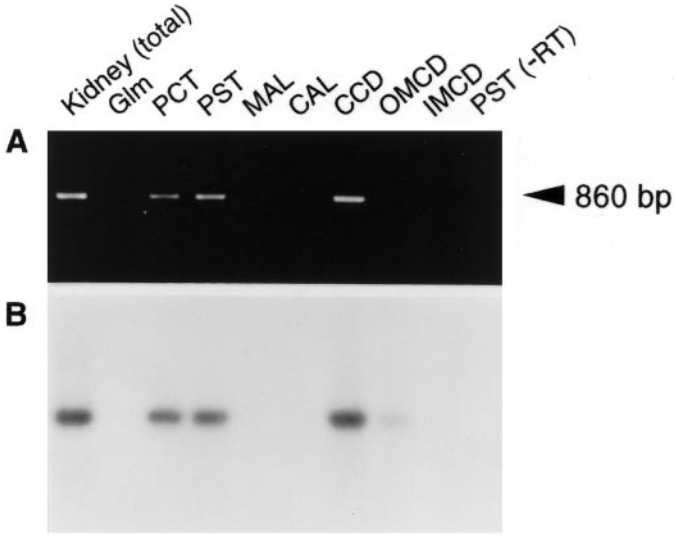

Fig. 4. Detection of OAT-K2 mRNA in microdissected renal nephron segments by RT-PCR on agarose gels (A) and autoradiograms of corresponding Southern blots (B). A, after microdissection, 5 glomeruli and 2 mm of each dissected tubule segment were reverse-transcribed, and the cDNA synthesized was amplified using a set of primers for OAT-K2 as described in the text. The PCR products were separated by electrophoresis through 1.5% agarose gels and stained with ethidium bromide. B, the agarose gels were transferred onto a nylon membrane and hybridized with the whole rat OAT-K2 cDNA as a probe at high stringency. Glm, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; MAL, medullary thick ascending limb; CAL, cortical thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

Tissue Distribution of OAT-K2 mRNA. Northern blot analysis of poly(A)$^+$ RNA from several rat tissues probed with whole OAT-K2 cDNA revealed that the OAT-K2 mRNA transcript was predominantly expressed in the rat kidney (Fig. 3A). A band with ~2.5 kb was detected under high-stringency conditions, and no hybridization signal was detected in mRNAs from any other tissues. The absence of the hybridizing mRNA species in the tissues was verified by detection of the GAPDH mRNA in each tissue (Fig. 3A). Because the whole OAT-K2 cDNA might hybridize to rat liver oatp1 and OAT-K1 mRNAs, the expression of OAT-K2 mRNA in rat tissues was further investigated by RT-PCR amplification. As shown in Fig. 3B, the PCR product with the expected size of 1038 bp for rat GAPDH was found in all the tissues examined. However, PCR amplification yielded product of expected size for rat OAT-K2 in both the kidney cortex and kidney medulla as well as in the OAT-K2 cRNA, but not in OAT-K1 cRNA and other tissues examined (Fig. 3B).

Renal tubular distribution of OAT-K2 mRNA. To obtain more detailed information about the localization of OAT-K2 mRNA in the kidney, we performed RT-PCR by using microdissected nephron segments. A band with 860 bp was detected in the proximal convoluted tubules, proximal straight tubules, and cortical collecting ducts (Fig. 4A). When the PCR procedure was carried out in the absence of reverse transcriptase, no band was detected from the proximal straight tubules, indicating that the PCR products originated from mRNA, not from genomic DNA. The Southern blots of the gels demonstrated that the OAT-K2 probe hybridized to the PCR products (Fig. 4B).

Functional Expression of OAT-K2 in *Xenopus* Oocytes. The transport function of OAT-K2 was investigated in oocytes by measuring the uptake of various anionic compounds, comparing with its homolog OAT-K1 (Fig. 5A). OAT-K1 and OAT-K2 stimulated the uptake of methotrexate and folate. The uptake of taurocholate and prostaglandin E_2 by the OAT-K2 cRNA-injected oocytes was also enhanced markedly, but not by the OAT-K1 cRNA-injected oocytes. Moreover, the taurocholate uptake in the OAT-K2-expressing oocytes was inhibited by the presence of unlabeled tau-

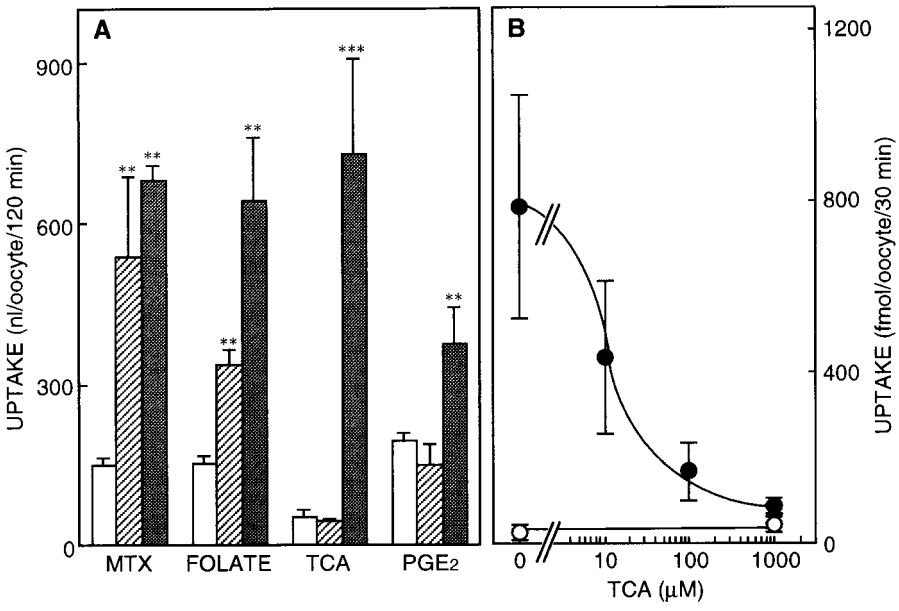

Fig. 5. Uptake of anionic drugs (A) and concentration-dependent inhibition of [^{3}H]taurocholate uptake by unlabeled taurocholate (B) in *Xenopus* oocytes. A, uptake by oocytes was assayed for 120 min at 25°C in incubation buffer containing [^{3}H]taurocholate (500 nM; 64.0 kBq/ml), [^{3}H]methotrexate (200 nM; 74.0 kBq/ml), [^{3}H]folate (200 nM; 74.0 kBq/ml), or [^{3}H]prostaglandin E_2 (5 nM; 33.5 kBq/ml) at 2 days after injection of 50 nl of water (open column), OAT-K1 cRNA (hatched column; 20 ng/oocyte) or OAT-K2 cRNA (shaded column, 20 ng/oocyte). B, uptake by oocytes was assayed for 30 min at 25°C in incubation buffer containing [^{3}H]taurocholate (1 μM; 128.0 kBq/ml) in the absence or presence of various concentrations of unlabeled taurocholate (10, 100, or 1000 μM) at 2 days after injection of 50 nl of water (○) or OAT-K2 cRNA (●; 40 ng/oocyte). Each column or point represents the mean ± S.E. of three experiments. Three oocytes were used for each experiment. **$p < 0.01$; ***$p < 0.001$, significant differences from each uptake value of water injected oocytes.

rocholate in a dose-dependent manner (IC_{50} = 10 μM), but not in water-injected oocytes (Fig. 5B).

Construction and Characterization of MDCK Cells Stably Transfected with OAT-K2 cDNA. To confirm the organic anion transport activity of OAT-K2 found in the oocyte expression system, we studied further characterization of OAT-K2 in the mammalian expression system by use of MDCK cells. Eleven transfectants expressing the OAT-K2 mRNA were isolated. Among these clones, single cells that showed the highest taurocholate transport activity were selected and named MDCK-OAT-K2. Figure 6 shows the intracellular accumulation of [^{3}H]taurocholate in the monolayers of MDCK-OAT-K2 and the MDCK-pBK cells. The accumulation from the apical side was much higher in MDCK-OAT-K2 than in mock-transfected MDCK-pBK monolayers. In contrast, the accumulation from the basolateral side in MDCK-OAT-K2 monolayers was comparable to that in MDCK-pBK monolayers. Next, we constructed the MDCK cells stably expressing OAT-K1 (MDCK-OAT-K1), as described above. The accumulation of methotrexate was measured by use of MDCK-OAT-K1 monolayers grown on membrane filters comparing with MDCK-pBK monolayers (from the apical side: MDCK-pBK, 14.3 ± 3.6; MDCK-OAT-K1, 162.5 ± 15.7 fmol/mg protein per 15 min, mean ± S.E. of three monolayers; from the basal side: MDCK-pBK, 53.8 ± 3.7; MDCK-OAT-K1, 76.0 ± 1.1 fmol/mg protein per 15 min, mean ± S.E. of three monolayers).

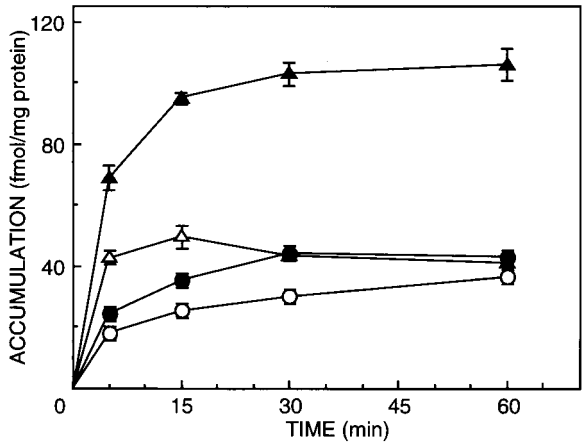

Fig. 6. Accumulation of [^{3}H]taurocholate by monolayers of MDCK-OAT-K2 cells. Monolayers of MDCK-OAT-K2 (●, ▲) or MDCK-pBK (○, △) grown on membrane filters were incubated for the specified period at 37°C with [^{3}H]taurocholate (250 nM, 32.0 kBq/ml) added to either the basolateral (●, ○; pH 7.4) or apical (▲, △; pH 7.4) side. Unlabeled uptake buffer without drugs was added to the opposite side (pH 7.4). After incubation, the radioactivity of solubilized cells was counted. Each value represents the mean ± S.E. of three monolayers.

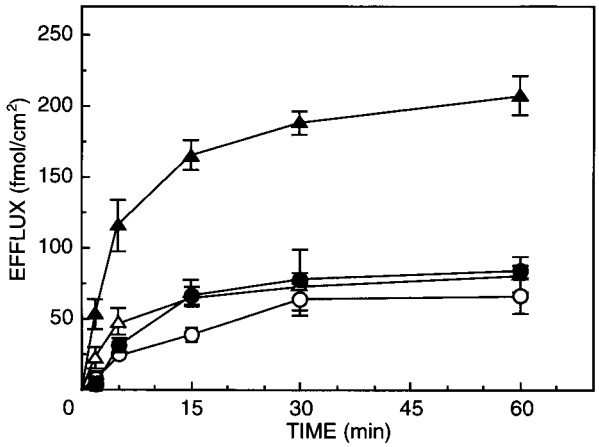

Fig. 7. Efflux of [^{3}H]taurocholate from monolayers of MDCK-OAT-K2 cells. [^{3}H]Taurocholate (250 nM, 32.0 kBq/ml) was added to both the apical and basolateral side of the monolayers of MDCK-OAT-K2 (●, ▲) or MDCK-pBK (○, △) grown on membrane filters. After incubation for 60 min at 37°C, the monolayers were washed and incubated for 60 min with unlabeled uptake buffer at 37°C. The efflux of taurocholate to the basolateral (●, ○; pH 7.4) or to the apical (▲, △; pH 7.4) side was measured. The data represent cumulative values. Each value represents the mean ± S.E. of three monolayers.

Figure 7 shows the efflux of taurocholate from MDCK-OAT-K2 and MDCK-pBK cells to the apical and basolateral side of the monolayers. MDCK-OAT-K2 and MDCK-pBK monolayers were preloaded from both sides with [^{3}H]taurocholate for 1 h and washed; then the [^{3}H]taurocholate released into each side of the monolayers was measured. The efflux of taurocholate to the apical side in the MDCK-OAT-K2 cells was much greater than that in the MDCK-pBK monolayers. In contrast, the efflux to the basolateral side in MDCK-OAT-K2 monolayers was comparable to that in the MDCK-pBK monolayers. Little intracellular taurocholate remained at the end of the incubation in each of the monolayers (<0.5% of time 0).

As shown in Fig. 8A, uptakes of methotrexate, taurocholate, and prostaglandin E_2 in the MDCK-OAT-K2 cells was enhanced markedly when compared with that in the MDCK-pBK cells. Figure 8B illustrates the taurocholate uptake in the MDCK-OAT-K2 cells as a function of the substrate concentration. The curve for the specific taurocholate uptake exhibited saturation kinetics with an apparent K_m value of 10.3 μM, corresponding to that in the oocyte expression system, and a V_{max} value of 30.1 pmol/mg protein/15 min, whereas the curve for the nonspecific uptake was almost linear over the concentration range examined.

To characterize the substrate specificity of the rat OAT-K2, we examined the [^{3}H]taurocholate uptake by MDCK-OAT-K2 cells under conditions of *cis*-inhibition. In Fig. 9A, sulfobromophthalein, probenecid, and indomethacin inhibited markedly the OAT-K2-mediated [^{3}H]taurocholate uptake. Methotrexate, furosemide, levofloxacin, and benzylpenicillin had relatively weak but significant inhibitory effects on the [^{3}H]taurocholate uptake. As shown in Fig. 9B, all of the bile acid derivatives had potent inhibitory effects on the [^{3}H]taurocholate uptake. Furthermore, steroids and related compounds remarkably inhibited the [^{3}H]taurocholate uptake (Fig. 9C). Cardiac glycosides, such as digoxin and ouabain, also had significant inhibitory effects on the [^{3}H]taurocholate uptake.

To determine whether indomethacin, digoxin, and testosterone would be transported by OAT-K2, the uptake of these drugs by MDCK-OAT-K2 and MDCK-pBK cells was measured. As summarized in Table 1, no enhanced uptake by MDCK-OAT-K2 cells was found for indomethacin, digoxin, and testosterone, relative to MDCK-pBK cells. As summarized in Table 2, replacement of Na^+ by *N*-methyl-D-glucamine had no significant effect on the taurocholate uptake by the MDCK-OAT-K2 cells. Furthermore, replacement of Cl^- with gluconate caused no significant change in the uptake.

Discussion

During the course of studies on the rat OAT-K1 (Saito et al., 1996), we have identified and characterized cDNA encoding OAT-K2, a new organic anion transporter, expressing specifically in the kidney of rats. The rat OAT-K2 has 91% amino acid identity with rat OAT-K1 transporter (Fig. 2). The amino acid sequences of OAT-K2 different from those of OAT-K1 showed appreciable identity with those of the other oatp transporters (Abe et al., 1998; Jacquemin et al., 1994; Noé et al., 1997). The amino acid sequence that is different from the other members of the oatp transporters may be OAT-K1 rather than OAT-K2.

RT-PCR for mRNA from the microdissected nephron segments with primers specific for OAT-K2 resulted in an expected length of PCR products. Similar to the mRNA distribution of OAT-K1 (Masuda et al., 1997b), OAT-K2 mRNA was highly expressed in the proximal straight tubules (Fig. 4). OAT-K2 mRNA was also highly expressed in the proximal convoluted tubules and the cortical collecting ducts, whereas the PCR products for OAT-K1 mRNA were detected at a fainter level in the proximal convoluted tubules and not detected in the cortical collecting ducts. These results suggest that OAT-K1 and OAT-K2 are both involved in the "renal organic anion transport system," especially in the proximal straight tubules.

The OAT-K2 mediates uptake of several anionic compounds, such as methotrexate, folate, taurocholate, and prostaglandin E_2 in OAT-K2-expressing oocytes, suggesting that the transporter has a broad range of substrate specificity different from that of OAT-K1 (Fig. 5A). The MDCK cells stably transfected with OAT-K2 also showed enhanced uptake of taurocholate, methotrexate, and prostaglandin E_2. Results from both the expression studies suggest that the OAT-K2 can recognize these structurally unrelated anionic substrates. Despite the highly conserved amino acid sequences between OAT-K1 and OAT-K2, the drug recognition by OAT-K2 appeared to be broad and different from that of OAT-K1. The OAT-K1 is incapable of mediating transport of either taurocholate or prostaglandin E_2 in oocytes (Fig. 5A), and both LLC-PK_1 (Saito et al., 1996) and MDCK (data not shown) cells stably transfected with OAT-K1. Although the original start codon of OAT-K1 still exists in OAT-K2, it is followed by a stop codon. The three insertions and three deletions of oligonucleotides in the 5′ noncoding nucleotide sequence of OAT-K2 in comparison with that of OAT-K1 would lead to an open reading frame shift, resulting in a shorter protein (Figs. 1 and 2). The six insertions and three deletions in the open reading frame of OAT-K2 in comparison with that of OAT-K1 would also lead to the frame shifts of the intracellular amino acid sequence of OAT-K2 between the predicted second and third transmembrane regions. Therefore, the OAT-K2 does not have the predicted first 4-transmembrane regions found in OAT-K1 and shows little homology with OAT-K1 along the intracellular sequences rich in charged amino acids between the predicted second and third transmembrane regions. These differences in the sequences might explain the substrate selectivity of OAT-K2. Similar findings were reported in the plasma membrane calcium-pumping ATPases (PMCAs) 4a and 4b. Despite the fact that both the nucleotide and amino acid sequences of PMCA 4b are highly conserved in those of PMCA 4a, the affinity of PMCA 4b for calmodulin is higher than that of PMCA 4a (Carafoli, 1994; Enyedi et al., 1994). Analyses of nucleotide sequences of OAT-K1 and OAT-K2 in genomic level should be further studied to clarify whether the nucleotide sequences coding these two transporters are the product of different genes and/or are due to alternate splicing.

Because the amino acid sequence of antigen peptide for the antiserum raised against the OAT-K1 (Masuda et al., 1997b) was identical with that of the OAT-K2, the anti-OAT-K1 antibody must have recognized not only OAT-K1, but also OAT-K2 protein. By Western blot analysis with the antiserum for rat OAT-K1, an immunoreactive protein was detected in the plasma membrane fractions of MDCK-OAT-K2 but not in those of MDCK-pBK cells (data not shown). The immunoreactive protein detected previously in brush-border membranes, but not in basolateral membranes by Western blotting with the antiserum for the rat OAT-K1 (Masuda et al., 1997b), could be composed of these two transporters. Therefore, the OAT-K2 transporter protein can be assumed

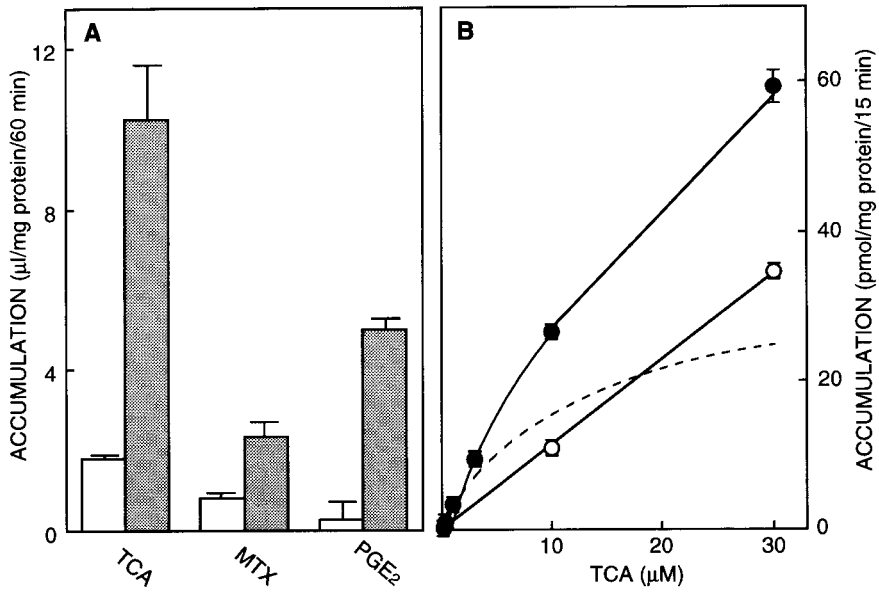

Fig. 8. Accumulation of anionic drugs (A) and concentration dependence of [^{3}H]taurocholate accumulation (B) by MDCK-OAT-K2 cells. A, MDCK-pBK (open column), or MDCK-OAT-K2 (shaded column) cells grown on 35-mm culture dishes were incubated for 60 min at 37°C with either [^{3}H]taurocholate (250 nM, 32.0 kBq/ml), [^{3}H]methotrexate (250 nM, 92.5 kBq/ml), or [^{3}H]prostaglandin E_2 (2.8 nM, 18.5 kBq/ml) (pH 7.4) in the absence (total) or presence (nonspecific) of 500 μM unlabeled taurocholate. After incubation, the radioactivity of solubilized cells was determined. The accumulation of each drug is expressed as specific uptake clearance derived from the difference between the total and nonspecific value. B, [^{3}H]taurocholate accumulation was measured at various concentrations (0.1–30 μM) for 15 min at 37°C (pH 7.4) in the absence (●) and presence (○) of 500 μM unlabeled taurocholate. Each column or point represents the mean ± S.E. of three experiments.

to be localized to the renal brush-border membranes as well as OAT-K1 (Masuda et al., 1997b) and oatp1 (Bergwerk et al., 1996).

Recently, we characterized the function of OAT-K1 by using the stable transfected LLC-PK$_1$ cells, suggesting that OAT-K1 mediates basolateral uptake of methotrexate (Saito et al., 1996) and is expressed with the apparent molecular mass of 70 kDa, corresponding to its calculated molecular mass of 74 kDa, in the plasma membrane fractions of the transfectant (Masuda et al., 1997a). However, Western blot analysis with the antiserum against rat OAT-K1 revealed that the transporter protein with an apparent molecular mass of 40 kDa was expressed exclusively in the brush-border membranes from rat kidney, suggesting that the rat OAT-K1 is localized in the renal brush-border membranes as a proteolytic processed molecule (Masuda et al., 1997b). In this study, we have found that the OAT-K1-mediated methotrexate transport was enhanced from the apical side, but not from the basolateral side, in MDCK-OAT-K1 cells. By Western blotting, an immunoreactive protein with an apparent molecular mass of 50 kDa comparable to that in rat renal brush-border membranes was detected in the plasma membrane fractions of MDCK-OAT-K1, but not in those of MDCK-pBK cells (data not shown). These results indicate that the rat OAT-K1 may be expressed functionally in apical membranes of the MDCK-OAT-K1 monolayers as a proteolytic processed molecule and mediate apical transport of methotrexate. The OAT-K2-mediated taurocholate uptake was also enhanced from the apical side, but not from the basolateral side (Fig. 6). Furthermore, the OAT-K2-mediated taurocholate efflux was enhanced across the apical membranes, but not across the basolateral membranes (Fig. 7). These results indicate that OAT-K2 is also localized to the apical membranes, but not to the basolateral membranes, in the transfectant. Therefore, the expression systems of the OAT-K1 and OAT-K2 transporters in the MDCK-transfectants should be useful in vitro models for studying mecha-

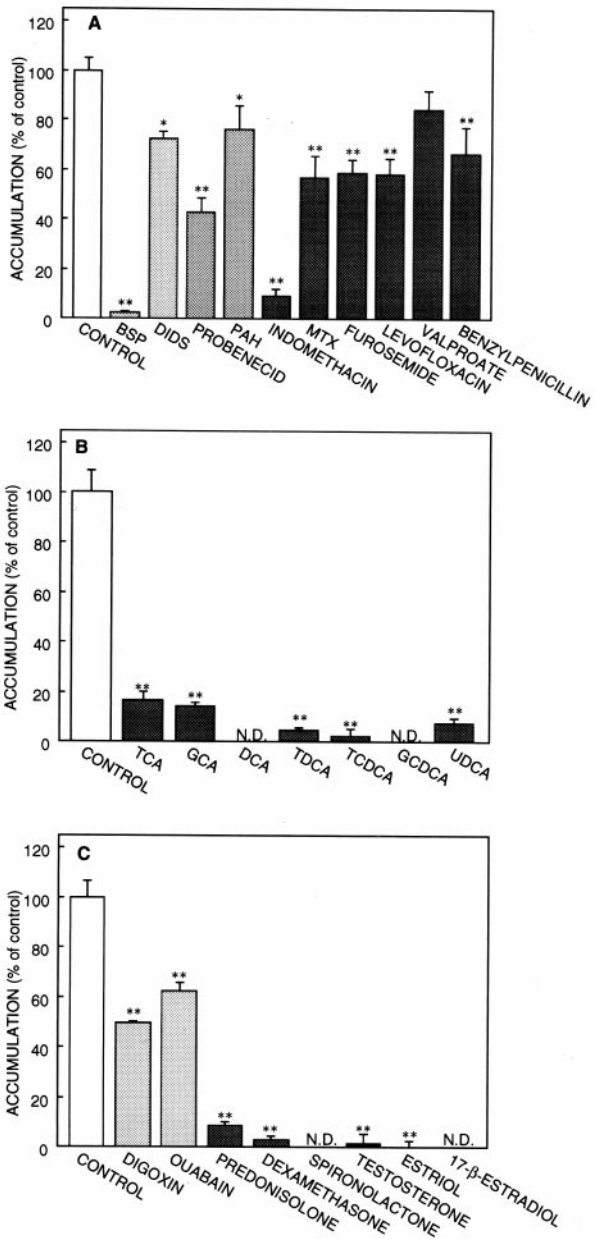

Fig. 9. Effect of various anionic drugs (A), bile acid analogs (B), and steroids (C) on [^{3}H]taurocholate accumulation by MDCK-OAT-K2 cells. [^{3}H]Taurocholate accumulation by MDCK-OAT-K2 cells (250 nM; 32.0 kBq/ml; 2 ml, pH 7.4) was measured for 15 min at 37°C in the absence (control) and presence of indicated compounds at a concentration of 100 μM in the absence (total) or presence (nonspecific) of 500 μM unlabeled taurocholate. After incubation, the radioactivity of solubilized cells was determined. Data are expressed as a percentage of the control value. BSP, sulfobromophthalein; PAH, *p*-aminohippurate; TCA, unlabeled taurocholate; GCA, glycocholate; DCA, deoxycholate; TDCA, taurodeoxycholate; TCDCA, taurochenodeoxycholate, GCDCA, glycochenodeoxycholate; UDCA, ursodeoxycholate. Each column represents the mean ± S.E. of three experiments. *$p < 0.05$; **$p < 0.001$, significant differences from control. N.D., not detected.

TABLE 1

Radiolabeled taurocholate, indomethacin, digoxin, and testosterone accumulation by MDCK-pBK and MDCK-OAT-K2 cells

[^{3}H]Taurocholate (500 nM; 64.0 kBq/ml), [^{14}C]indomethacin (3 μM; 2.5 kBq/ml), [^{3}H]digoxin (250 nM; 148.0 kBq/ml), and [^{3}H]testosterone (22 nM; 74.0 kBq/ml) accumulation by MDCK-OAT-K2 cells (2 ml; pH 7.4) was measured for 60 min at 37°C in the absence (total) or presence (nonspecific) of 500 μM unlabeled taurocholate. After incubation, the radioactivity of solubilized cells was determined. Accumulation of each drug is expressed as the specific uptake clearance derived from the difference between the total and nonspecific value. Each value represents the mean ± S.E. of three experiments.

Drugs	Uptake clearance	
	MDCK-pBK	MDCK-OAT-K2
	μl/mg protein/60 min	
[^{3}H]Taurocholate	1.83 ± 0.09	10.25 ± 1.35***
[^{14}C]Indomethacin	−0.92 ± 1.75	1.24 ± 1.18
[^{3}H]Digoxin	−0.38 ± 0.15	−0.27 ± 0.23
[^{3}H]Testosterone	−0.04 ± 0.02	0.02 ± 0.04

*** $p < 0.001$, significant differences from MDCK-pBK.

TABLE 2

Effect of extracellular Na^+ and Cl^- depletion on [^{3}H]taurocholate accumulation in MDCK-OAT-K2 cells

MDCK-OAT-K2 cells were preincubate at 37°C for 20 min either in normal, Na^+-free, or Cl^--free medium as described in the text and incubated at 37°C for 5 min with taurocholate (250 nM, 32.0 kBq/ml) in the absence (total) or presence (nonspecific) of 500 μM unlabeled taurocholate (2 ml; pH 7.4). Each value represents the mean ± S.E. of three experiments.

Treatments	[^{3}H]Taurocholate accumulation	
	fmol/mg protein/5 min	% of Control
Control	597.1 ± 31.3	100
Na^+-free	610.3 ± 2.7	102
Cl^--free	684.4 ± 31.6	115

nisms involved in transport functions and membrane localizations.

Similar to methotrexate uptake by OAT-K1 and sulfobromophthalein uptake by oatp1, the taurocholate uptake via OAT-K2 was dependent on neither extracellular Na^+ nor Cl^-, suggesting that the process of OAT-K2-mediated taurocholate uptake is a facilitated transport process, not a secondary active transport process (Table 2). The exact transport mechanisms, including direct coupling with other inorganic ions and/or dependence on the membrane potential of the OAT-K2, remain to be elucidated.

In the kidney, filtered taurocholate is reabsorbed by an Na^+-dependent transport system (K_m = 330 μM) in the proximal tubule (Wilson et al., 1981). The OAT-K2-mediated taurocholate uptake (K_m = 10.3 μM) was strongly suppressed by the presence of several bile acid derivatives in the MDCK-OAT-K2 cells (Fig. 9B). OAT-K2 might affect the renal bile acid reabsorption process in an Na^+-independent manner as a high affinity component. In addition, transport studies with isolated renal membrane vesicles have contributed to the understanding of secretory mechanisms for organic anions, i.e., the dicarboxylate/*p*-aminohippurate exchanger in basolateral membranes (Shimada et al., 1987) and the membrane potential-dependent transport system in brush-border membranes (Ohoka et al., 1993). Most recently, the renal basolateral-type multispecific organic anion transporter, OAT-1/ROAT1, has been cloned and suggested to be the basolateral membrane dicarboxylate/*p*-aminohippurate exchanger (Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997). However, the brush-border membrane organic anion transporters have not been fully elucidated. The ATP-dependent multispecific organic anion export pump, Mrp2/cMOAT, was identified in the bile canalicular membranes of liver (Paulusma et al., 1996) and also found to be localized in the brush-border membranes of renal proximal tubules by immunohistochemical study (Schaub et al., 1997). Mrp2/cMOAT transporter may contribute to cellular detoxification and to the secretion of anionic substances, most of which are conjugates, from the blood into urine. A series of bile acids, steroids, and structurally unrelated organic anions were recognized by OAT-K2 (Fig. 9), and endogenous taurocholate and prostaglandin E_2 were transported via OAT-K2 (Figs. 5A and 8A). Prostaglandin E_2, which derives principally from renal synthesis in medullary interstitial cells, collecting duct cells and blood vessels (Bonvalet et al., 1987; Dunn and Hood, 1977), was secreted into the urine, probably by transport processes in the proximal tubules that are sensitive to probenecid (Haylor et al., 1990). Furthermore, OAT-K2 was suggested to function as a bidirectional organic anion transporter in the apical membranes (Figs. 6 and 7). Therefore, it can be assumed that the OAT-K2 participates physiologically in the tubular detoxification of various endogenous anions and anionic xenobiotics across the brush-border membranes, thereby contributing to organic anion secretion.

In conclusion, cDNA encoding a new organic anion transporter protein, OAT-K2, was isolated from the kidney of rats. The predominant expression of the OAT-K2 mRNA in the kidney and its functional properties suggest that the OAT-K2 contributes to renal secretion and/or reabsorption of hydrophobic anionic compounds.

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