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Expression and Functional Characterization of Rat Organic Anion Transporter 3 (rOat3) in the Choroid Plexus

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ABSTRACT

We reported previously that an efficient efflux system for benzylpenicillin (PCG) is located on the choroid plexus (CP). In this study, we investigated the involvement of rat organic anion transporter 1 (rOat1; *Slc22a6*) and rOat3 (*Slc22a8*) in the uptake of PCG and *p*-aminohippurate (PAH) by the CP. Western blot analysis indicates the expression of rOat3, but not rOat1, on the CP, and immunohistochemical staining shows that rOat3 is localized on the brush border membrane of the choroid epithelial cells. PCG and PAH were found to be taken up by isolated rat CP, with K_m values of 111 and 354 μM, respectively. A mutual inhibition study suggests that the same transporter is responsible for the uptake of PCG and PAH by isolated rat CP. This was confirmed by examining the effect of organic anions and cimetidine on their uptake. Estradiol-17β-glucuronide and cimetidine were found to be selective inhibitors of rOat3. The inhibition constants of the inhibitors including estradiol-17β-glucuronide and cimetidine were comparable for the uptake of PAH and PCG by isolated rat CP. In addition, these values were also comparable with those for rOat3, but not with those for rOat1. These results suggest that rOat3 is mainly responsible for the uptake of PCG and PAH by isolated rat CP, and it functions as one of the detoxification systems on the CP by removing its substrates from the cerebrospinal fluid.

The choroid plexus (CP), located in the lateral, third, and fourth ventricles, is the site of the production of cerebrospinal fluid (CSF), and it is also responsible for the homeostasis of the environment surrounding the brain by keeping the composition of the CSF constant (Segal, 2001). It is well established that the CP acts as a barrier between the CSF and the circulating blood (Suzuki et al., 1997; Spector, 2000; Gao and Meier, 2001; Ghersi-Egea and Strazielle, 2001; Haselbach et al., 2001; Kusuhara and Sugiyama, 2001); this is achieved partly by the tight monolayer of the choroid epithelial cells and partly by the detoxification systems, such as metabolism in the CP, and efflux transport (Suzuki et al., 1997; Spector, 2000; Gao and Meier, 2001; Ghersi-Egea and Strazielle, 2001; Haselbach et al., 2001; Kusuhara and Sugiyama, 2001). Organic anions, such as estradiol-17β-glucuronide ($E_2$17βG), benzylpenicillin (PCG), and cefodizime (β-lactam antibiotics) in the CSF are actively transported from the CSF into the circulating blood across the CP (Suzuki et al., 1997; Spector 2000; Haselbach et al., 2001; Kusuhara and Sugiyama 2001). We demonstrated previously that the CP is the site of elimination of PCG from the CSF (Suzuki et al., 1997). A series of uptake studies using isolated rat CP revealed that an anion exchanger is involved in the uptake of PCG by the CP (Suzuki et al., 1997). Mutual inhibition studies provided kinetic evidence to suggest that this transporter is also responsible for the uptake of a variety of compounds, such as cefodizime (a β-lactam antibiotic), dideoxyinosine (a nucleoside analog), fleroxacin (a new quinolone antibiotic), fluorescein, and phenol red, by the CP (Suzuki et al., 1997; Hakvoort et al., 1998). However, the responsible transporter remains unidentified (Suzuki et al., 1997; Nishino et al., 1999; Kusuhara and Sugiyama, 2001).

The present study was carried out to investigate whether rat organic anion transporter 1 (rOat1; *Slc22a6*) and/or rOat3 (*Slc22a8*) is expressed on the CP and is responsible for the uptake of PCG by the CP, if expressed. rOat1 has been isolated from rat kidney by expression cloning using *Xenopus laevis* oocytes (Sekine et al., 1997). The transport via rOat1 is driven by an outward concentration gradient of dicarboxylates, and its substrates include organic anions such as *p*-aminohippurate (PAH), a typical organic anion for the renal organic anion transporter, PCG, and nucleoside analogs (Inui et al., 2000; Sekine et al., 2000; Van Aubel et al., 2000; Dresser et al., 2001). Transcription of mouse Oat1 (mOat1) in the CP was detected between embryonic days 12 and 16 (e12

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ABBREVIATIONS: CP, choroid plexus; CSF, cerebrospinal fluid; $E_2$17βG, estradiol-17β-glucuronide; PAH, *p*-aminohippurate; Oat, organic anion transporter; Oatp, organic anion transporter polypeptide; m, mouse; r, rat; PCG, benzylpenicillin; TBS-T, Tris-buffered saline/Tween 20; BBM, brush border membrane; BLM, basolateral membrane.

and e16) of embryo development by in situ hybridization, but it was not detected in the newborn and adult CP (Pavlova et al., 2000). In contrast, Pritchard et al. (1999) demonstrated that the properties of 2,4-dichlorophenoxyacetate uptake by the CP are similar to those of rOat1 and suggested that the latter is involved in transport. The K_i value of PCG for rOat1 is significantly larger (1.7 mM; Jariyawat et al., 1999; Hasegawa et al., 2002) than the K_m value for the uptake of PCG by isolated rat CP (approximately 80 μM), suggesting that rOat1 may account for a low-affinity component, if expressed.

We have isolated rOat3, a third member of the organic anion transporter family, from rat brain using homology screening (Kusuhara et al., 1999). Northern blot analysis indicates its expression in the liver, kidney, brain, and, weakly, in the eye (Kusuhara et al., 1999). The transcript of mOat3 was detected in the CP from e14 of embryo development, but the expression of mOat3 in the adult CP is unclear (Pavlova et al., 2000). According to our preliminary experiments, PCG is a good substrate for rOat3, with a K_m value similar to that for the uptake of PCG by the CP, and a fragment corresponding to rOat3 was amplified by the use of reverse transcription-polymerase chain reaction using cDNA prepared from adult rat CP, prompting us to hypothesize that rOat3 is responsible for the uptake of PCG by the CP. In this study, we examined the expression and localization of rOat1 and rOat3 in the CP and compared kinetic parameters for the uptake of PAH and PCG by isolated rat CP and by LLC-PK1 cells expressing rOat1 and rOat3.

Experimental Procedures

Materials. [^{3}H]PAH and [^{14}C]PAH (4.08 Ci/mmol and 50.4 mCi/mmol, respectively) and [^{3}H]H_2O were purchased from PerkinElmer Life Sciences (Boston, MA), and [^{3}H]PCG and [^{14}C]PCG (19.0 Ci/mmol and 56.0 mCi/mmol, respectively) were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All cell culture media and reagents were obtained from Invitrogen (Carlsbad, CA), except for fetal bovine serum, which was obtained from Cansera International (Rexdale, Ontario, Canada). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Antiserum and Western Blot Analysis. Antiserum against rOat1 and rOat3 were raised in rabbits against a synthetic peptide consisting of the 16 carboxyl-terminal amino acids of rOat1 and rOat3, respectively. Membrane fractions were prepared from LLC-PK1 cells expressing rOat1 and rOat3 and from kidney, as described previously (Sugiyama et al., 2001; Hasegawa et al., 2002). The membrane fractions and CP isolated from the lateral ventricles were diluted with loading buffer (BioLabs, Hertfordshire, UK). These specimens were boiled for 3 min and then loaded onto a 10% SDS-polyacrylamide electrophoresis gel with a 4.4% stacking gel. For Western blotting, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham) using a blotter (Trans-Blot; Bio-Rad, Hercules, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with anti-rOat3 serum (dilution, 1:1000). The membrane was then allowed to bind a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham) diluted to 1:5000 in TBS-T for 1 h at room temperature followed by washing with TBS-T.

Immunofluorescence Study. Frozen sections from male Sprague-Dawley rats purchased from Japan SLC (Shizuoka, Japan) were prepared after fixing in acetone at 4°C for 10 min. Nonspecific protein binding was blocked by incubation with Nonspecific Staining Blocking Reagent (DAKO, Carpinteria, CA). Sections were incubated with anti-rOat3 antibodies (1:200) for 1 h at room temperature, washed three times with TBS-T, and subsequently incubated with the secondary antibodies labeled with fluorescein isothiocyanate for 30 min at room temperature and mounted in VECTASHIELD Mounting Medium with propidium iodide (Vector Laboratories, Burlingame, CA). The specificity of the antibody reaction was verified by negative controls that were incubated with antiserum that had been blocked with the antigenic peptide.

Uptake Studies in cDNA-Transfected LLC-PK1 Cells. The cDNA transfectants (LLC-PK1 cells expressing rOat1 and rOat3) were established previously, and all the procedures have been described in detail (Sugiyama et al., 2001). Cells were seeded on a 12-well dish (BD Biosciences, Franklin Lakes, NJ) at a density of 1.2×10^5 cells/well and were cultured for 3 days. Sodium butyrate (5 mM) was added to the culture medium to induce expression of the transporter 24 h before starting the experiments (Sugiyama et al., 2001). Uptake was initiated by adding medium containing radiolabeled ligands after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. This buffer consists of 142 mM NaCl, 23.8 mM Na_2CO_3, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4. The uptake was terminated at a designed time by adding ice-cold Krebs-Henseleit buffer, and cells were kept overnight in 500 μl of 1 N NaOH for lysis. The radioactivity associated with the cells and medium was determined by liquid scintillation counting after adding 2 ml of scintillation fluid (Hionic-Fluor; Packard Instrument Co., Meriden, CT) to the vials. The remaining 20-μl portions of cell lysate were used to determine the protein concentration by the Lowry method, with the use of bovine serum albumin as a standard.

Uptake of PAH and PCG by Isolated Rat CP. Male Sprague-Dawley rats weighing 250 to 300 g were purchased from Japan SLC. The uptake of [^{14}C]PCG and [^{14}C]PAH by isolated rat CP was examined by the use of centrifugal filtration as described in detail previously (Suzuki et al., 1986). The CP was isolated from the lateral ventricles and incubated at 37°C for 1 min in 500 μl of artificial CSF, consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, pH 7.3, equilibrated with 95% O_2/5% CO_2. Radiolabeled ligands, with or without inhibitors, were added to initiate uptake. The tissue-to-medium concentration ratio of [^{14}C]PCG and [^{14}C]PAH was calculated with [^{3}H]H_2O as a cell water space marker to correct for the adherent water space. The ^{3}H and ^{14}C activity in the specimens was determined in a liquid scintillation spectrophotometer (LS6000SE; Beckman Coulter, Inc., Fullerton, CA).

Kinetic Analyses. Kinetic parameters were obtained using the Michaelis-Menten equation: $v = V_{max} \times S/(K_m + S) + P_{dif} \times S$, where v is the uptake rate of the substrate (in picomoles per minute per milligram of protein or picomoles per minute per milliliter of tissue), S is the substrate concentration in the medium (micromolar), K_m is the Michaelis-Menten constant (micromolar), and V_{max} is the maximum uptake rate (in picomoles per minute per milligram of protein or picomoles per minute per milliliter of tissue). P_{dif} represents the uptake clearance corresponding to the nonsaturable component (milliliters per minute per milligram of protein or milliliters per minute per milliliter of tissue). To obtain the kinetic parameters, the equation was fitted to the uptake velocity using a MULTI program (Yamaoka et al., 1981). The input data were weighted as the reciprocals of the observed values and the Damping Gauss Newton Method algorithm was used for fitting. Inhibition constants (K_i) of several compounds were calculated by assuming competitive inhibition. An inhibitory effect was investigated by examining the uptake of PCG by rOat3 at 5 min and that of PAH by rOat1 at 1 min, as well as examining the uptake of PCG and PAH by CP at 5 min.

Results

Expression of rOat1 and rOat3 on the CP. The expression of rOat1 and rOat3 in their cDNA-transfected cells, CP, and kidney was examined by Western blot analysis (Fig. 1). Immunoreactive protein was detected at approximately 63 and 50 kDa in LLC-PK1 cells expressing rOat3 by rOat3 antiserum and at 63 kDa in the CP, respectively (Fig. 1a, lanes 2 and 3). The molecular mass of rOat3 detected in the kidney was slightly greater than that in the CP (Fig. 1a, lanes 3 and 4). These bands were abolished when preabsorbed antiserum for rOat3 was used (Fig. 1a, lanes 5–8), suggesting that the positive bands were specific for the antigen peptide. rOat1 antiserum detected a single band at a molecular mass of 69 kDa in LLC-PK1 cells expressing rOat1 and the kidney (Fig. 1b, lanes 1 and 4). Although a faint band was detected by rOat1-antiserum in the CP (Fig. 1b, lane 3), this was ascribed to nonspecific binding because the band was even detected by preabsorbed antiserum (Fig. 1b, lane 7).

Localization of rOat3 on rat CP. The basal surface of the choroid epithelial cells is apposed to a capillary bed, whereas the brush border surface, covered with microvilli, faces the CSF. As shown in Fig. 2, the positive signal of rOat3 is localized on the brush border membrane (BBM) of the choroid epithelial cells. Preincubating the antiserum of rOat3 with antigen abolished the signal (data not shown). Positive signals were observed in the CP when a frozen section of rat brain was incubated with rOat1 antiserum (data not shown). However, it was not abolished by preabsorption of the antiserum for rOat1 (data not shown), suggesting that the positive signal observed on the CP is caused by nonspecific binding.

Uptake of PAH and PCG by LLC-PK1 Cells Expressing rOat1 and rOat3. The time profiles of the uptake of PAH and PCG by rOat1 and rOat3 are shown in Fig. 3. The intracellular accumulation of PAH and PCG by LLC-PK1 cells expressing rOat3 was significantly greater than that by vector-transfected cells (Fig. 3, a and b). Because the uptake of PAH and PCG by LLC-PK1 cells expressing rOat3 increased linearly for up to 5 min of incubation (Figs. 3a), the uptake of PAH and PCG at 5 min was used for further studies. The uptake of PAH and PCG by LLC-PK1 cells expressing rOat3 was saturated at higher substrate concentration. Kinetic analysis showed that the K_m and V_{max} values and the uptake clearance for the nonsaturable component of PCG uptake by rOat3 were 82.6 ± 31.5 μM, 172 ± 67 pmol/min/mg of protein, and 0.758 ± 0.123 μl/min/mg of protein, respectively (Fig. 4a; Table 1). Although the uptake of PAH by LLC-PK1 cells expressing rOat3 was significantly greater than that by vector-transfected cells, the saturable component accounts for 30% of the total uptake by LLC-PK1 cells expressing rOat3, indicating that the intrinsic transport activity of PAH by rOat3 is quite small compared with that of PCG (approximately 10-fold smaller).

A significant increase in the uptake of PAH was observed in LLC-PK1 cells expressing rOat1 (Fig. 3d). The concentration-dependence of the uptake of PAH by rOat1 determined at 1 min is shown in Fig. 3d, which indicates that the uptake

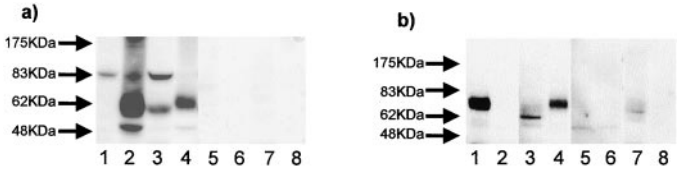

Fig. 1. Expression of rOat1 and rOat3 on the cDNA transfectants and CP. a, expression of rOat3 in crude membrane fractions prepared from LLC-PK1 cells expressing rOat1 and rOat3 (lanes 1 and 2; 5 μg/lane), isolated CP (lane 3; 3.3 μg/lane), and plasma membrane fraction from the kidney (lane 4; 5 μg/lane) was examined using rOat3 antiserum against carboxy terminus of rOat3. They are separated by SDS-polyacrylamide gel electrophoresis (10% separating gel). Immunoreactivity was completely abolished by pretreatment of rOat3 antiserum with antigen (lanes 5–8, the same order for lanes 1–4). b, expression of rOat1 in crude membrane fractions prepared from LLC-PK1 cells expressing rOat1 and rOat3 (lanes 1 and 2; 1.3 μg/lane), isolated CP (lane 3; 3.3 μg/lane), and plasma membrane fraction from the kidney (lane 4; 5 μg/lane) was examined. They are separated by SDS-polyacrylamide gel electrophoresis (10% separating gel). Immunoreactivity was completely abolished by pretreatment of rOat1 antiserum with antigen (lanes 5–8, the same order for lanes 1–4).

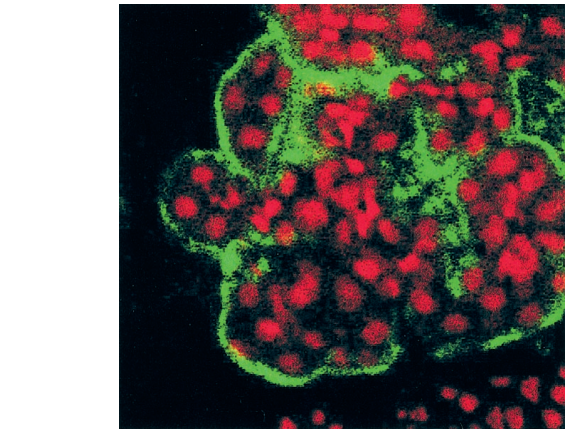

Fig. 2. Localization of rOat3 in the rat CP. Cry sections of brain from male Sprague-Dawley rats were incubated with rOat3 antiserum. Nuclei were stained with propidium iodide. The BBM of the CP was stained with rOat3-antiserum (green fluorescence).

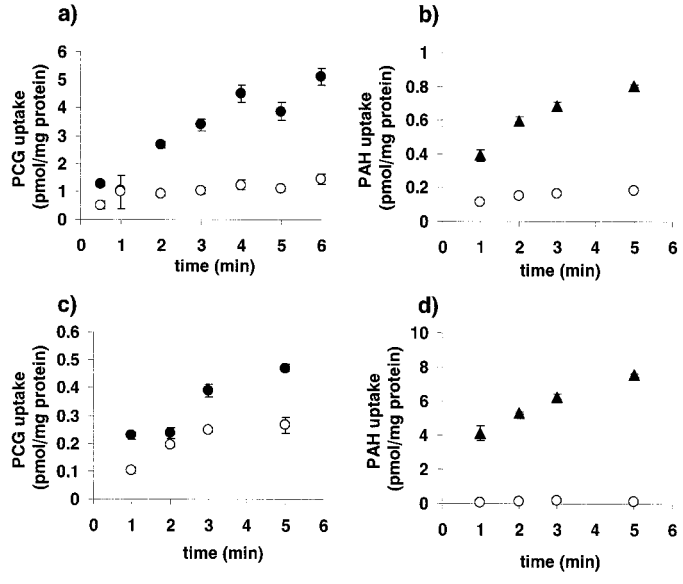

Fig. 3. Uptake of [^{3}H]PAH and [^{3}H]PCG by LLC-PK1 cells expressing rOat3 and rOat1. The uptake of [^{3}H]PCG and [^{3}H]PAH by rOat3 (a, b) and rOat1 (c, d) was examined at 37°C. ▲, uptake by LLC-PK1 cells expressing rOat1; ○, uptake by LLC-PK1 cells expressing rOat3; ○, uptake by vector-transfected cells. Uptake was initiated by adding radiolabeled ligand (0.3 μM [^{3}H]PCG or 0.2 μM [^{3}H]PAH) and was terminated at designated times by adding ice-cold buffer.

of PAH by rOat1 consists of one saturable and one nonsaturable component (Fig. 4b). The K_m and V_{max} values of PAH by rOat1 were determined to be 47.0 ± 5.2 μM and 1330 ± 110 pmol/min/mg of protein, respectively. The uptake clearance corresponding to the nonsaturable component was 0.745 ± 0.080 μl/min/mg of protein, which was comparable with the uptake of PAH by vector transfected cells. A slight increase in the uptake of PCG by rOat1-expressed cells was observed compared with the uptake by vector-transfected cells (2.35 and 1.32 μl/mg protein at 5 min, respectively; $p < 0.01$). The uptake of PAH by LLC-PK1 cells expressing rOat1 at the substrate concentration sufficient to saturate the uptake was comparable with that of vector-transfected cells.

Effect of Organic Anions and Cimetidine on the Uptake of PCG and PAH by rOat1 and rOat3. The effect of organic anions, such as estrone sulfate, $E_2$17βG, and probenecid, and an organic cation, cimetidine, on rOat1-mediated PAH uptake and rOat3-mediated PCG uptake was examined (Fig. 6). The K_i values of these compounds were obtained by assuming competitive inhibition, and they are summarized in Table 1. Probenecid and estrone sulfate were the most potent inhibitors for rOat3, whereas cimetidine and $E_2$17βG were moderate inhibitors (Fig. 6; Table 1). Because cimetidine and $E_2$17βG do not inhibit the uptake by rOat1 at all (Fig. 6, Table 1) (Sugiyama et al., 2001), the involvement of rOat1 can be discriminated by examining their inhibitory effect on the uptake of PAH and PCG by isolated rat CP.

Uptake of PCG and PAH by Isolated Rat CP. The time profiles of the uptake of PAH and PCG by isolated rat CP are shown in Fig. 5a. The uptake of PAH and PCG by isolated rat CP increased linearly for up to 5 min of incubation. Their uptake at 5 min was used to examine the concentration-dependence and the effect of various inhibitors. Kinetic analysis revealed that the uptake of PCG and PAH consists of one saturable and one nonsaturable component (Fig. 5). The K_m and V_{max} values for the uptake of PCG and PAH by isolated rat CP were determined to be 111 ± 19 μM and 224 ± 32 pmol/min/μl of tissue, and 354 ± 84 μM and 154 ± 34 pmol/min/μl of tissue, respectively. The uptake clearance corresponding to the nonsaturable component was 0.246 ±

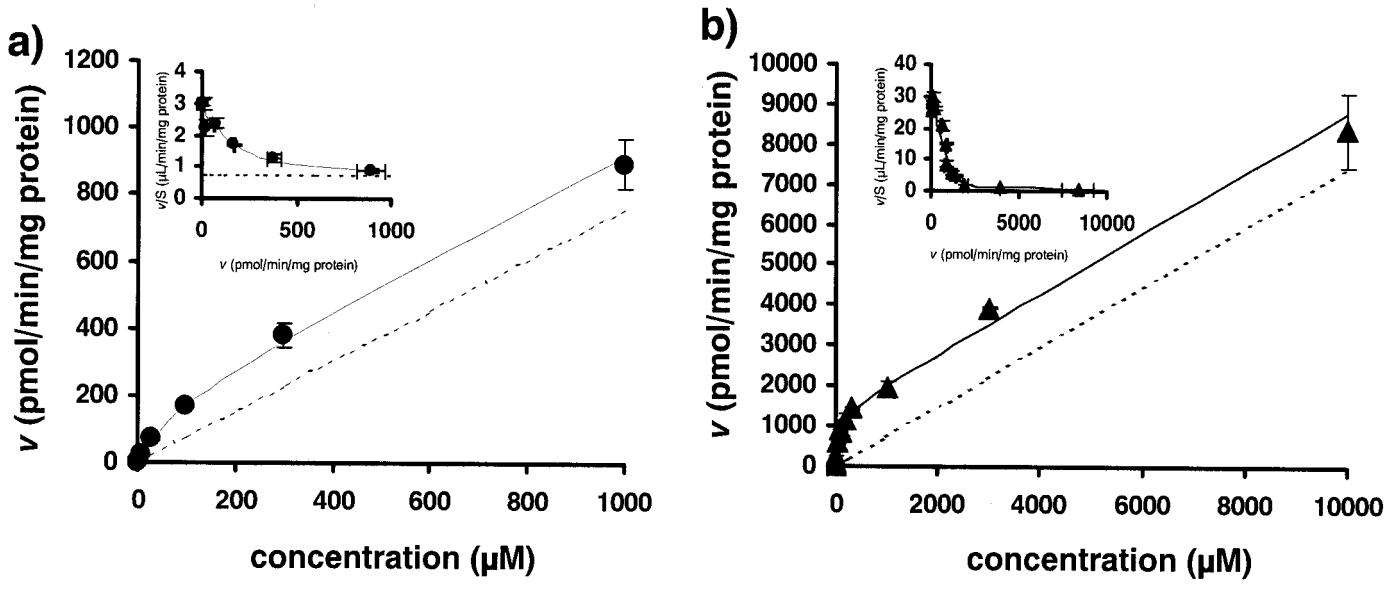

Fig. 4. Concentration dependence for rOat3-mediated [^{3}H]PCG uptake and rOat1-mediated [^{3}H]PAH uptake. The concentration-dependence of rOat3-mediated [^{3}H]PCG uptake (a) and rOat1-mediated [^{3}H]PAH uptake (b) is shown. Kinetic analyses revealed that the uptake of [^{3}H]PCG and [^{3}H]PAH consists of one saturable and one nonsaturable component. Michaelis-Menten constants were obtained by nonlinear regression analysis, and the solid and broken lines represent the fitted line and the uptake corresponding to the nonsaturable component, respectively. Each point represents the mean ± S.E. (n = 3).

TABLE 1

K_i and K_m values for the uptake of PCG and PAH by isolated rat CP, rOat3 and rOat1

The effect of estrone sulfate, cimetidine, probenecid, $E_2$17βG, PAH and PCG was examined on the uptake of PCG and PAH by isolated rat CP, and PCG and PAH by rOat3 and rOat1. These K_i and K_m values were determined by nonlinear regression analysis as described under *Experimental Procedures*. Data are taken from Figures 4 to 6 and from Sugiyama et al. (2001). Values represent the mean ± S.D. (n = 3).

	K_i and K_m			
	CP (PCG)	CP (PAH)	rOat3 (PCG)	rOat1 (PAH)
			μM	
Estrone sulfate	22.3 ± 18.3	34.8 ± 10.6	9.10 ± 5.23	N.D.
Cimetidine	44.4 ± 9.4	51.0 ± 7.0	46.8 ± 15.1	>1000
Probenecid	2.29 ± 0.66	3.29 ± 0.30	4.43 ± 0.89	31.0 ± 7.9[a]
$E_2$17βG	33.0 ± 6.8	26.8 ± 6.8	35.6 ± 18.3	>300[a]
PAH	406 ± 184	354 ± 84[b]	398 ± 154	47.0 ± 5.2[b]
PCG	111 ± 19[b]	69.6 ± 21.1	82.6 ± 31.5[b]	800 ± 361

N.D., not determined.
[a] Sugiyama et al. (2001).
[b] K_m.

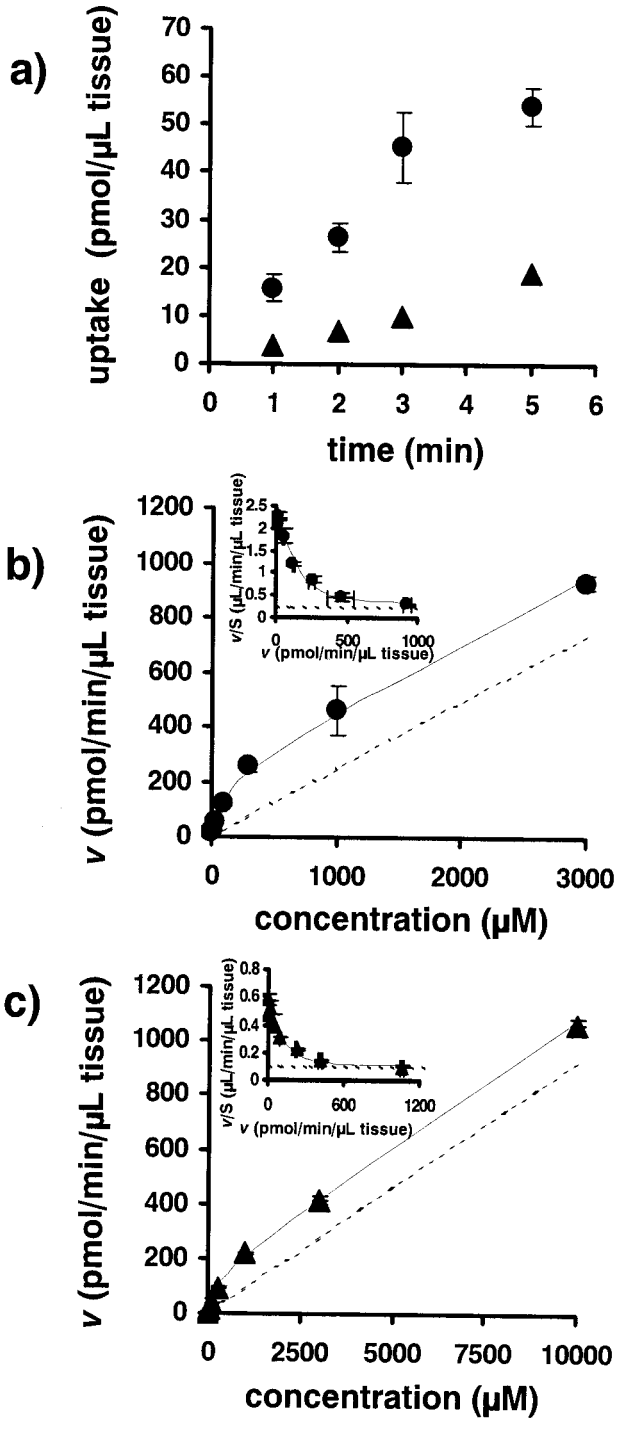

Fig. 5. Uptake of [^{14}C]PCG and [^{14}C]PAH by isolated rat CP and concentration-dependence for their uptake by isolated rat CP. The CP was isolated from the lateral ventricles. The uptake of [^{14}C]PCG and [^{14}C]PAH by isolated rat CP was examined by centrifugal filtration as described under *Experimental Procedures*. The tissue-to-medium concentration ratio of [^{14}C]PCG and [^{14}C]PAH was calculated with [^{3}H]H_2O as a cell water space marker and was corrected for the adherent water space. ●, uptake of [^{14}C]PCG by isolated CP; ▲, uptake of [^{14}C]PAH by isolated CP. The concentration-dependence of the uptake of [^{14}C]PCG (b) and [^{14}C]PAH (c) by isolated rat CP is shown. The uptake determined at 5 min with different substrate concentrations of PAH and PCG was used for the calculation. The solid and broken lines represent the fitted line and the uptake corresponding to the nonsaturable component, respectively. Each point represents the mean ± S.E. (n = 3).

0.025 μl/min/μl of tissue and 0.0927 ± 0.0104 μl/min/μl of tissue, respectively. The uptake of PCG by isolated rat CP under linear conditions (V_{max}/K_m) was 5-fold greater than that of PAH.

A mutual inhibition study was carried out to examine whether the same transporter is responsible for the uptake of PCG and PAH by isolated rat CP. As shown in Table 1, the K_m values of PCG and PAH for their uptake by isolated rat CP were comparable with the K_i values for the uptake of PAH and PCG, suggesting that PCG and PAH exhibit mutually competitive inhibition (Table 1).

Effect of Organic Anions and Cimetidine on the Uptake of PCG and PAH by Isolated Rat CP. The effect of the inhibitors, described previously, on the uptake of PAH and PCG by isolated rat CP was examined. Probenecid is the most potent inhibitor of the uptake of PAH and PCG by isolated rat CP, whereas estrone sulfate, cimetidine, and $E_2$17βG are moderate inhibitors (Fig. 6). The inhibition constants of these inhibitors are summarized in Table 1. The K_i values were comparable for the uptake of PAH and PCG by isolated rat CP (Table 1, Fig. 7). In addition, the K_i and K_m values were also comparable with those for rOat3 but not with those for rOat1 (Table 1, Fig. 7).

Discussion

In this study, we demonstrated the expression and localization of rOat3 on isolated rat CP and the involvement of rOat3 in the uptake of PCG and PAH by isolated rat CP. Because Pritchard et al. (1999) proposed the functional involvement of rOat1 in the uptake of 2,4-dichlorophenoxyacetic acid by the CP, we also examined the expression of rOat1 on the CP.

The expression of rOat1 and rOat3 on the CP was studied with the use of Western blot analysis. A band was detected in the CP by rOat3 antiserum (Fig. 1a), and its molecular mass was slightly smaller than that in the kidney. This may be caused by a difference in the degree of glycosylation. Immunohistochemical staining indicates that rOat3 is located on the BBM of the CP (Fig. 2). Because the BBM of the CP faces the CSF, the localization of rOat3 suggests its involvement in removing exogenous and endogenous compounds from the CSF. On the other hand, rOat1 antiserum failed to detect any band in the CP (Fig. 1b, lane3), whereas it detected a single band in LLC-PK1 cells expressing rOat1 and in the kidney (Fig. 1b, lanes 1 and 4). These results indicate that the expression of rOat1 on the CP is either very low or zero.

The localization of rOat3 on the CP differs from that in the kidney, where it is localized on the basolateral membrane (BLM) of the proximal tubules (Cha et al., 2001; Hasegawa et al., 2002). The same tissue-specific localization was also reported in the case of Na^+/K^+ ATPase and reduced folate carrier, which exhibit the same pattern as rOat3, i.e., the BLM of the kidney and the BBM of the CP (Marrs et al., 1993; Wang et al., 2001). In addition, rOat1-green fluorescent fusion protein, transfected exogenously into the CP, was localized on the BBM of the CP (Pritchard et al., 1999), although it is localized on the BLM of *Fundulus heteroclitus* proximal tubules when it is exogenously transfected (Sweet et al., 1999). The presence of a tissue-specific membrane-sorting mechanism has been considered, although the molecular mechanism remains to be clarified.

According to the transport study using cDNA transfected cells, PAH and PCG are good probes to detect the transport activity by rOat1 and rOat3, respectively. Significant uptake of PCG and PAH was observed in isolated rat CP (Fig. 5). Both the absolute value of the uptake and the K_m value for the uptake of PCG by isolated rat CP were comparable with previously reported values (Suzuki et al., 1987). The uptake of PAH was 5-fold smaller than that of PCG (Fig. 5). As summarized in Table 1, the results from the mutual inhibition study suggest that, kinetically, PAH and PCG share the same uptake system on the CP. This was also supported by comparing the K_i values of inhibitors, including a selective inhibitor of rOat3, of the uptake of PAH and PCG, which were found to be similar (Table 1, Fig. 7). In addition, these parameters (K_m and K_i values) were also similar to those for rOat3, but not for rOat1 (Table 1, Fig. 7). In conjunction with the results of the Western blot analysis and immunohistochemical staining, we conclude that rOat3 is mainly responsible for the uptake of PCG and PAH by the CP.

Rat organic anion transporting polypeptide 1 (rOatp1; *Slc21a1*) has been demonstrated to be localized on the BBM of the CP (Angeletti et al., 1997; Nishino et al., 1999), it has been isolated from rat liver by expression cloning using *X. laevis* oocytes, and it has been shown to be a multispecific transporter for amphipathic organic anions such as $E_2 17\beta G$ and bile acids (Bossuyt et al., 1996; Muller and Jansen 1997). There is an overlap in substrates between rOatp1 and rOat3 [e.g., $E_2 17\beta G$, estrone sulfate, ochratoxin A, and pravastatin (Eckhardt et al., 1999; Kusuhara et al., 1999; Sugiyama et al., 2001; Hasegawa et al., 2002)], suggesting that some common ligands may be removed from the CSF by both rOatp1 and rOat3. The contribution of rOatp1 and rOat3 to the total uptake of organic anions by the CP should be evaluated in further studies to reveal the role of these organic anion transporters in the elimination of organic anions from the CSF. Taking their substrate specificity into consideration, we hypothesize that rOatp1 is responsible mainly for the uptake of amphipathic organic anions by the CP, whereas rOat3 is mainly responsible for the uptake of less hydrophobic organic anions. Consequently, the uptake system for organic anions

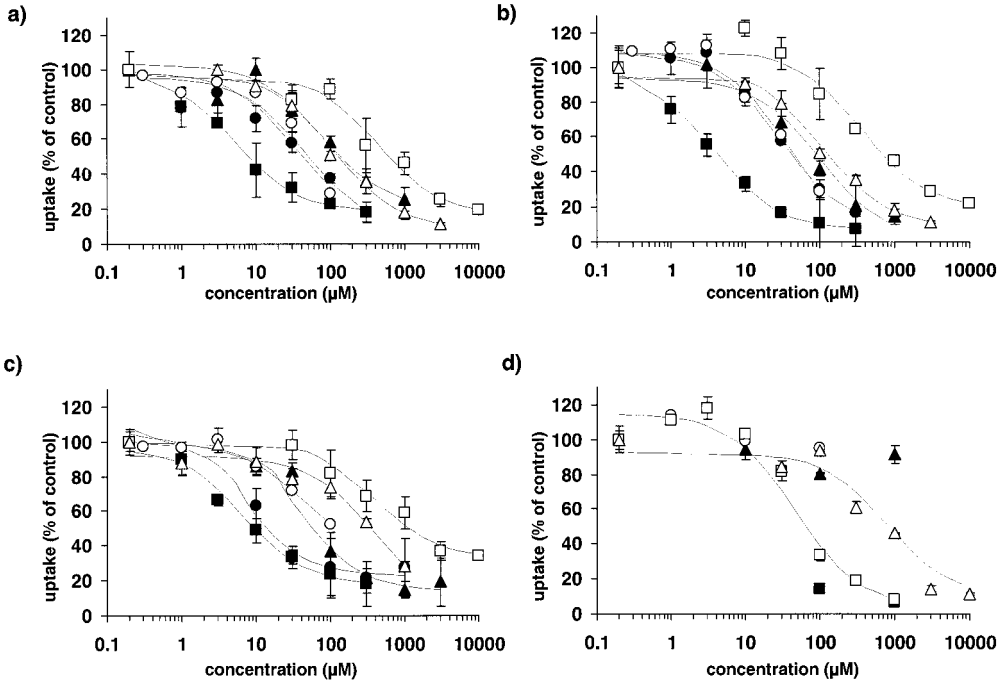

Fig. 6. Inhibitory effect of organic anions and cimetidine on the uptake of PAH by rOat1, and the uptake of PCG by rOat3 and isolated rat CP. The uptake of [^{14}C]PCG by CP (a), [^{14}C]PAH by CP (b), [^{3}H]PCG by rOat3 (c), and [^{3}H]PAH by rOat1 (d) was determined in the presence and absence of inhibitors at the concentrations indicated. ●, estrone sulfate; ▲, cimetidine; ■, probenecid; ○, $E_2 17\beta G$; □, PCG; △, PAH. The inhibition constants (K_i) of these compounds were calculated assuming competitive inhibition. The solid lines represent the fitted line obtained by nonlinear regression analysis. The details of fitting are described under *Experimental Procedures*. Each point represents the mean ± S.E. (n = 3).

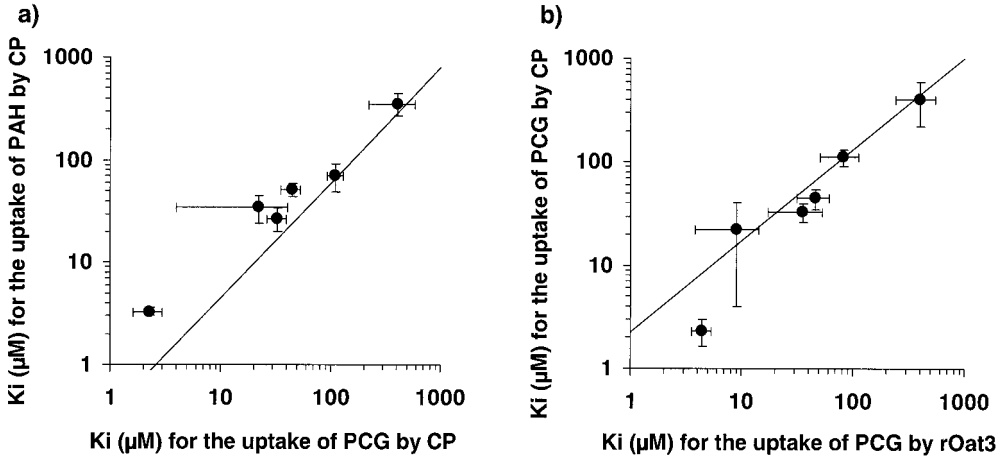

Fig. 7. Correlation of K_m and K_i values for the uptake of PAH and PCG by isolated rat CP, and for the uptake of PCG by rOat3 and isolated rat CP. Data are taken from Table 1. Correlation of K_m and K_i values for the uptake of PCG by rOat3 and CP (a) and that of PAH and PCG by CP (b). The solid lines represent the regression line of the K_m and K_i values for the uptake of PAH and PCG by isolated rat CP (a; $r = 0.956$, $p < 0.01$), and the uptake of PCG by isolated rat CP and by rOat3 (b; $r = 0.985$, $p < 0.01$).

located on the BBM of the CP may cover a wide range of organic anions and may remove their substrates efficiently from the CSF.

To excrete xenobiotics from the CSF into the circulating blood, efflux transporters are required on the BLM of the CP (Suzuki et al., 1997; Wijnholds et al., 2000;Gao and Meier, 2001). However, transport across the BLM has not yet been fully characterized. rMrp1 and rOatp2 have been demonstrated to be expressed on the BLM of choroid epithelial cells (Gao et al., 2000; Wijnholds et al., 2000). Because rOatp2 is a bidirectional transporter (Li et al., 2000), it is possible that the uptake and excretion of organic anions on the BLM is mediated by rOatp2, although its function on the BLM of the CP has not yet been demonstrated (Gao et al., 1999). The role of Mrp1 on the CP as an excretion mechanism has been demonstrated by comparing the CSF concentration of etoposide between Mdr1a/Mdr1b double knockout mice and Mdr1a/Mdr1b/Mrp1 triple knockout mice (Wijnholds et al., 2000). The CSF concentration of etoposide significantly increased in Mdr1a/Mdr1b/Mrp1 triple knockout mice compared with Mdr1a/Mdr1b double knockout mice. The contribution of these transporters to the elimination of organic anions needs to be examined in future studies.

In conclusion, our studies have shown that rOat3 is mainly responsible for the uptake of PCG and PAH by the CP. It is one of the uptake mechanisms for the removal of organic anions from the CSF together with rOatp1.

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References

Angeletti RH, Novikoff PM, Juvvadi SR, Fritschy JM, Meier PJ, and Wolkoff AW (1997) The choroid epithelium is the site of the organic anion transport protein in the brain. *Proc Natl Acad Sci USA* **94:**283–286.

Bossuyt X, Muller M, and Meier PJ (1996) Multispecific amphipathic substrate transport by an organic anion transporter of human liver. *J Hepatol* **25:**733–738.

Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, and Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59:**1277–1286.

Dresser M, Leabman M, and Giacomini K (2001) Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters. *J Pharm Sci* **90:**397–421.

Eckhardt U, Schroeder A, Stieger B, Hochli M, Landmann L, Tynes R, Meier PJ, and Hagenbuch B (1999) Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. *Am J Physiol* **276:**G1037–G1042.

Gao B, Hagenbuch B, Kullak-Ublick GA, Benke D, Aguzzi A, and Meier PJ (2000) Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J Pharmacol Exp Ther* **294:**73–79.

Gao B and Meier PJ (2001) Organic anion transport across the choroid plexus. *Microsc Res Tech* **52:**60–64.

Gao B, Stieger B, Noe B, Fritschy JM, and Meier PJ (1999) Localization of the organic anion transporting polypeptide 2 (Oatp2) in capillary endothelium and choroid plexus epithelium of rat brain. *J Histochem Cytochem* **47:**1255–1264.

Ghersi-Egea JF and Strazielle N (2001) Brain drug delivery, drug metabolism, and multidrug resistance at the choroid plexus. *Mirosc Res Tech* **52:**83–88.

Hakvoort A, Haselbach M, and Galla HJ (1998) Active transport properties of porcine choroid plexus cells in culture. *Brain Res* **795:**247–256.

Hasegawa M, Kusuhara H, Sugiyama D, Ito K, Ueda S, Endou H, and Sugiyama Y (2002) Functional involvement of rat organic anion transporter 3 (rOat3; *Slc22a8*) in the renal uptake of organic anions. *J Pharmacol Exp Ther* **300:**746–753.

Haselbach M, Wegener J, Decker S, Engelbertz C, and Galla HJ (2001) Porcine choroid plexus epithelial cells in culture: regulation of barrier properties and transport process. *Microsc Res Tech* **52:**137–152.

Inui KI, Masuda S, and Saito H (2000) Cellular and molecular aspects of drug transport in the kidney. *Kidney Int* **58:**944–958.

Jariyawat S, Sekine T, Takeda M, Apiwattanakul N, Kanai Y, Sophasan S, and Endou H (1999) The interaction and transport of β-lactam antibiotics with the cloned rat renal organic anion transporter 1. *J Pharmacol Exp Ther* **290:**672–677.

Kusuhara H, Sekine T, Ustunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y, and Endou H (1999) Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. *J Biol Chem* **274:**13675–13680.

Kusuhara H and Sugiyama Y (2001) Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier. *Drug Discov Today* **6:**150–156.

Li L, Meier PJ, and Ballatori N (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Mol Pharmacol* **58:**335–340.

Marrs JA, Napolitano EW, Murphy-Erdosh C, Mays RW, Reichardt LF, and Nelson WJ (1993) Distinguishing roles of the membrane-cytoskeleton and cadherin mediated cell-cell adhesion in generating different Na^+, K^+-ATPase distributions in polarized epithelia. *J Cell Biol* **123:**149–164.

Muller M and Jansen PLM (1997) Molecular aspects of hepatobiliary transport. *Am J Physiol* **272:**G1285–G1303.

Nishino J, Suzuki H, Sugiyama D, Kitazawa T, Ito K, Hanano M, and Sugiyama Y (1999) Transepithelial transport of organic anions across the choroid plexus: possible involvement of organic anion transporter and multidrug resistance-associated protein. *J Pharmacol Exp Ther* **290:**289–294.

Pavlova A, Sakurai H, Leclercq B, Beier DR, Yu ASL, and Nigam SK (2000) Developmentally regulated expression of organic ion transporters NKT (OAT1), OCT1, NLT (OAT2), and Roct. *Am J Physiol* **278:**F635–F643.

Pritchard JB, Sweet DH, Miller DS, and Walden R (1999) Mechanism of organic anion transport across the apical membrane of choroid plexus. *J Biol Chem* **274:**33382–33387.

Segal MB (2001) Transport of nutrients across the choroid plexus. *Microsc Res Tech* **52:**28–48.

Sekine T, Cha SH, and Endou H (2000) The multispecific organic anion transporter (OAT) family. *Pfluegers Arch Eur J Physiol* **440:**337–350.

Sekine T, Watanabe N, Hosoyamada M, Kanai Y, and Endou H (1997) Expression cloning and characterization of a novel multispecific organic anion transporter. *J Biol Chem* **272:**18526–18529.

Spector R (2000) Drug transport in the mammalian central nervous system: multiple complex systems. A critical analysis and commentary. *Pharmacology* **60:**58–73.

Sugiyama D, Kusuhara H, Shitara Y, Abe T, Meier PJ, Sekine T, Endou H, Suzuki H, and Sugiyama Y (2001) Characterization of the efflux transport of 17β-estradiol-D-17β-glucuronide from the brain across the blood-brain barrier. *J Pharmacol Exp Ther* **298:**316–322.

Suzuki H, Sawada Y, Sugiyama Y, Iga T, and Hanano M (1986) Transport of cimetidine by the rat choroid-plexus in vitro. *J Pharmacol Exp Ther* **239:**927–935.

Suzuki H, Sawada Y, Sugiyama Y, Iga T, and Hanano M (1987) Transport of benzylpenicillin by the rat choriod plexus in vivo. *J Pharmacol Exp Ther* **242:**660–665.

Suzuki H, Terasaki T, and Sugiyama Y (1997) Role of efflux transport across the blood-brain barrier and blood cerebrospinal fluid barrier on the disposition of xenobiotics in the central nervous system. *Adv Drug Deliv Rev* **25:**257–285.

Sweet DH, Miller DS, and Pritchard JB (1999) Localization of an organic anion transporter-GFP fusion construct (rROAT1-GFP) in intact proximal tubules. *Am J Physiol* **276:**F864–F873.

Van Aubel RA, Masereeuw R, and Russel FG (2000) Molecular pharmacology of renal organic anion transporters. *Am J Physiol* **279:**F216–F232.

Wang Y, Zhao R, Russell RG, and Goldman ID (2001) Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. *Biochim Biophys Acta* **1513:**49–54.

Wijnholds J, deLange EC, Scheffer GL, van den Berg DJ, Mol CA, van der Valk M, Schinkel AH, Scheper RJ, Breimer DD, and Borst P (2000) Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. *J Clin Invest* **105:**279–285.

Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmokinetic analysis program (MULTI) for microcomputer. *J Pharmacobiodyn* **4:**879–885.

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