

**Title : Contribution of organic anion transporter 3 (*Slc22a8*) to the elimination of *p*-aminohippuric acid and benzylpenicillin across the blood-brain barrier**

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**Running title : Role of rOat3 at the BBB**

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Abbreviations: BBB, blood-brain barrier; BCEC, brain capillary endothelial cells; BCSFB, blood-cerebrospinal fluid barrier; BEI, brain efflux index; CSF, cerebrospinal fluid; DHEAS, dehydroepiandrosterone sulfate; E<sub>1</sub>S, estrone-3-sulfate; E<sub>2</sub>17βG, 17β-estradiol-D-17β-glucuronide; IS, indoxyl sulfate; Oat, organic anion transporter; Oatp, organic anion transporting polypeptide; Oct, organic cation transporter; PAH, *para*-aminohippuric acid; PCG, benzylpenicillin; P-gp, P-glycoprotein; TCA, taurocholic acid

## Abstract

The role of rat Organic anion transporter 3 (*Slc22a8*) in the efflux transport at the blood-brain barrier (BBB) was characterized. The expression of rOat1, rOat2, and rOat3 in the brain capillary endothelial cells (BCEC) was examined using RT-PCR analysis, which showed that there was no expression of rOat1 or rOat2, but moderate expression of rOat3. The expression of rOat3 in the BCEC was further confirmed by Western blotting. Immunohistochemical staining showed that rOat3 is located on the abluminal and, possibly, luminal membrane of the BCEC. The contribution of rOat3 to the efflux of *para*-aminohippuric acid (PAH) and benzylpenicillin (PCG), substrates of rOat3, from the cerebrum into the blood circulation across the BBB was evaluated using the Brain Efflux Index (BEI) method. PAH and PCG were eliminated from the cerebrum with rate constants of  $0.039 \text{ min}^{-1}$  and  $0.043 \text{ min}^{-1}$ , respectively, and the elimination was saturated at high substrate concentrations. Taking account of the dilution in the brain, the  $K_m$  values for the elimination of PAH and PCG were estimated to be  $168 \mu\text{M}$  and  $29 \mu\text{M}$ , respectively. The efflux of PAH and PCG across the BBB was inhibited in a dose-dependent manner by unlabeled PCG and PAH, respectively. The  $K_i$  value of PAH for the efflux of PCG was  $106 \mu\text{M}$  and that of PCG for the efflux of PAH was  $58 \mu\text{M}$ . These values were comparable with their  $K_m$  values, suggesting that they share the same efflux mechanism at the BBB. Furthermore, cimetidine and pravastatin, which are also substrates and inhibitors of rOat3, significantly inhibited the efflux of PAH and PCG from the cerebrum. These results suggest that rOat3 is responsible for the elimination of PAH and PCG from the brain across the BBB.

## Introduction

The BBB is well known to restrict the passage of xenobiotics from the systemic circulation into the brain parenchyma (Suzuki et al., 1997; Pardridge, 1999; Lee et al., 2001). The molecular basis of the barrier function involves highly developed tight junctions between the BCEC, metabolic enzymes, and efflux transporters located on the plasma membrane of the BCEC. Due to the tight junctions, it is unlikely that exogenous or endogenous compounds reach the brain via a paracellular route. Thus, the transcellular route is the major pathway for the exchange of compounds between the brain parenchyma and the circulating blood.

It has been shown that there is a positive correlation between the lipophilicity and the permeability coefficient across the BBB (Levin, 1980). Thus, certain drugs with high lipophilicity can pass into the brain through the BBB. However, there are some drugs which achieve a lower brain distribution than expected from their lipophilicity. This has been explained by the efflux transport at the BBB. Indeed, antitumor agents such as doxorubicin and vincristine exhibit poor brain penetration despite their high lipophilicity (Levin, 1980). The unexpectedly low permeability of these drugs across the BBB can be accounted for by P-glycoprotein-mediated efflux at the BBB.

Employing a microinjection technique (BEI method), the efflux transport systems for organic anions at the BBB have been investigated. It has been shown that transporters are involved in the efflux of amphipathic organic anions such as taurocholic acid (TCA) and conjugated steroids such as  $17\beta$ -estradiol-D- $17\beta$ -glucuronide ( $E_217\beta G$ ), estrone-3-sulfate ( $E_1S$ ), and dehydroepiandrosterone sulfate (DHEAS), and a hydrophilic organic anion, PAH, across the BBB (Kitazawa et al., 1998; Asaba et al., 2000; Hosoya et

al., 2000; Sugiyama et al., 2001). A member of the hepatic organic anion transporter family, Organic anion transporting polypeptide (Oatp) 2 has been identified on both the luminal and abluminal membrane of the BCEC (Gao et al., 1999). Since Oatp2 accepts amphipathic organic anions described above as substrates (Reichel et al., 1999), it has been a candidate transporter responsible for the elimination of amphipathic organic anions from the brain. However, PAH is not a substrate of Oatp2 (Reichel et al., 1999), suggesting the involvement of additional organic anion transporter(s) in the extrusion of PAH from the cerebrum across the BBB.

Since PAH is a typical substrate of the Oat family, which consist of three members in rats (rOat1 to rOat3) (Inui et al., 2000; Sekine et al., 2000; Dresser et al., 2001), the efflux transport of PAH across the BBB may be mediated by the member(s) of this family. Among the Oat family in rats, the expression of rOat1 and rOat3 in the brain has been reported. rOat1 has been isolated from rat kidney by the expression cloning method (Sekine et al., 1997). Functional characterization has shown that the properties of rOat1 are consistent with those of the classical organic anion/dicarboxylate exchanger responsible for multispecific organic anion transport at the basolateral membrane of renal proximal tubules (Shimada et al., 1987). Northern blot analysis revealed that rOat1 is expressed predominantly in the kidney and weakly in the brain (Sekine et al., 1997). rOat3 was first isolated from the brain using the RT-PCR cloning method, and Northern blot analysis revealed that rOat3 mRNA is expressed in the liver, brain, kidney, and weakly in the eye (Kusuhara et al., 1999). rOat3 accepts amphipathic organic anions such as E<sub>2</sub>17βG and E<sub>1</sub>S as well as PAH as its substrates (Kusuhara et al., 1999; Sugiyama et al., 2001). In the brain, rOat3 has been shown to be expressed in the choroid plexus

(Nagata et al., 2002) as well as in the brain capillaries (Ohtsuki et al., 2002). In the choroid plexus, rOat3 is responsible for the uptake of PAH and PCG from the cerebrospinal fluid (CSF) (Nagata et al., 2002). This information suggests the possibility that rOat1 and rOat3 in the brain may be responsible for the elimination of PAH from the cerebrum.

In the present study, the expression of rOat1, rOat2, and rOat3 at the BBB was investigated using RT-PCR analysis, Western blotting, and immunohistochemistry. The contribution of rOat3 to the efflux of organic anions such as PAH and PCG from the brain into the blood circulation was evaluated using the BEI method.

## Materials and Methods

**Chemicals** [ $^3\text{H}$ ]PAH (4.54 Ci/mmol) and [ $^{14}\text{C}$ ]carboxyl-inulin (2.5 mCi/g) were purchased from PerkinElmer Life Science Products (Boston, MA). [ $^3\text{H}$ ]PCG (19.5 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Unlabeled PAH was purchased from Sigma (St. Louis, MO), and unlabeled PCG and cimetidine were from Wako Pure Chemical Industries (Osaka, Japan). Unlabeled pravastatin was kindly donated by Sankyo (Tokyo, Japan). Ketamine hydrochloride was purchased from Sankyo (Tokyo, Japan). Xylazine and ketamine hydrochloride were used as anesthetics. All other chemicals were commercially available, of reagent grade, and used without further purification.

**Animals** Sprague-Dawley male rats (supplied by Japan SLC) weighing 220 to 250 g were used throughout this study and had free access to food and water.

**Capillary isolation** The brain capillary-enriched fraction was isolated from rat cerebrum as described previously with some modifications (Boado and Pardridge, 1991). Briefly, pieces of gray matter were gently homogenized in three volumes (v/w) of buffer A (103 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 15 mM HEPES, pH 7.4) then the homogenate was centrifuged (5800 g) after adding dextran (15%). The resulting pellet was resuspended in buffer B (103 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 15 mM HEPES, 25 mM  $\text{NaHCO}_3$ , 10 mM D-glucose, 1 mM  $\text{Na}^+$ -Pyruvate, 0.5% (w/v) BSA, pH 7.4) and filtered through a 200  $\mu\text{m}$  nylon mesh. The filtrate was passed over a column of glass beads and washed with 500 ml buffer B. The capillaries adhering to the beads were collected by gentle agitation followed by centrifugation at 500 g. All steps in the isolation procedure were carried out

at 4 °C in pregassed (95% O<sub>2</sub>-5% CO<sub>2</sub>) solutions.

**RT-PCR analysis** Total RNA from the rat brain capillary-enriched fraction, kidney, and liver was prepared by a single-step guanidium thiocyanate procedure using ISOGEN (NIPPON GENE, Toyama, Japan). The RNA was then reverse-transcribed using a random-hexamer primer (Takara, Japan). cDNA fragments were amplified by PCR with the cDNA prepared from the brain capillary-enriched fraction, kidney, and liver as a template using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Inc., USA). The following primers were used to amplify rOat1, rOat2, and rOat3 cDNA: forward primer, 5'-ctgaactacctgcagacag-3' and reverse primer, 5'-atggagagacagaggaagag-3' for rOat1, forward primer, 5'-agacctgtccgaacatctc-3' and reverse primer, 5'-gagtgcagtaagtccaatc-3' for rOat2, and forward primer, 5'-gacctggagttatctcatgc-3' and reverse primer, 5'-ccggaacaagtcagataagc-3' for rOat3. PCR was performed according to the following procedure: 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min ; 40 cycles. The sequences of the amplified fragments were confirmed by DNA sequencing using a DNA sequencer (Model 377 DNA sequencer; Perkin Elmer, Foster City, CA).

**Antiserum** Anti-rOat3 antiserum was raised in rabbits against a synthetic peptide consisting of the 16 carboxyl-terminal amino acids of rOat3 coupled to keyhole limpet hemocyanine at its carboxyl-terminal via an additional tyrosine.

**Western blotting** The brain capillaries were resuspended in ice cold 1% Triton-100/PBS (500 µl for 10 mg capillary proteins) and stored at 4 °C for 20 min. The sample was centrifuged at 15000 g for 15 min and the supernatant was used for Western blotting. The rat kidney plasma membrane fraction was prepared by the standard procedure (Nakajima et al., 2000). 10 µg brain capillary proteins and 1 µg rat kidney plasma membrane



proteins were electrophoresed on 10% SDS-polyacrylamide gel with a 4.4% stacking gel. Separated proteins were transferred to a polyvinylidene difluoride membrane using a blotter at 15 V for 1 hr. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 hr at room temperature. After washing three times with TBS-T for 5 min, the membrane was incubated with anti-rOat3 antiserum (1 : 500 dilution) at room temperature for 1 hr. After washing, the membrane was allowed to bind a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) diluted 1 : 5000 in TBS-T for 1 hr at room temperature and detected using ECL plus (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Deglycosylation of rOat3** N-Linked carbohydrate groups were cleaved from the rOat3 protein in the brain capillaries and the kidney plasma membrane using PNGase F (*N*-glycosidase F) (BioLab, New England). Aliquots of 10  $\mu$ g capillary proteins or 3  $\mu$ g kidney proteins were added to a 10- $\mu$ l volume of 50 mM sodium phosphate buffer (pH 7.5) containing 1% NP-40 and 200 units/ $\mu$ l *N*-glycosidase F, with a final SDS concentration of 0.5%. As a control, distilled water was substituted for *N*-glycosidase F in an otherwise identical reaction mixture. After 1 hr incubation at 37 °C, the samples were subjected to Western blotting as described above.

### **Immunohistochemical staining**

**Double immunostaining with antibodies to rOat3 and P-glycoprotein** A 7-week-old male Sprague-Dawley rat was perfused with PBS for 5 min, followed by 2% paraformaldehyde (PFA) for 5 min. Then, the brain was removed and stored in 2% PFA for 1 hr at 4 °C. Before sectioning, the brain was infused with 18% sucrose. Cryostat

sections (10  $\mu\text{m}$  in thickness) were fixed in methanol at  $-20\text{ }^{\circ}\text{C}$  for 10 min, washed three times with PBS, and blocked with 1% BSA/PBS at room temperature for 15 min. Double staining was performed incubating the brain slices with anti-rOat3 antiserum (1 : 100 dilution in 1% BSA/PBS) and C219 (1 : 40 dilution in 1% BSA/PBS) at  $4\text{ }^{\circ}\text{C}$  overnight. After washing with PBS, sections were incubated with secondary antibodies (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG, diluted to 1 : 100, Molecular Probes) for 1 hr and mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA).

**Double immunostaining with antibodies to rOat3 and glial fibrillary acidic protein**

**(GFAP)** Cryostat sections were prepared as described above. To activate the antigen, sections were treated with microwaves for 5 min in 10 mM citric acid buffer, pH 6.0. Then, sections were fixed in ethanol at  $4\text{ }^{\circ}\text{C}$  for 30 min, followed by acetone at room temperature for 1 min. After washing with PBS, sections were incubated with 0.5% Triton-X/PBS at room temperature for 15 min, washed again, and blocked with 1% BSA/PBS for 15 min. Double-staining was performed as described above using anti-rOat3 antiserum (1 : 100) and monoclonal mouse anti-human GFAP (Dako, Japan) (1 : 25) both diluted in 1% BSA, 0.2% Triton X-100/PBS at  $4\text{ }^{\circ}\text{C}$  overnight. After washing with PBS, sections were incubated with secondary antibodies (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG, diluted to 1 : 100) for 1 hr and mounted in VECTASHIELD Mounting Medium.

**BBB efflux study** The efflux of test compounds from the brain after microinjection into the cerebral cortex was investigated using the BEI method as described previously

(Kakee et al., 1996). Briefly, rats were anesthetized with intramuscular doses of ketamine (125 mg/kg) and xylazine (1.22 mg/kg) and placed in a stereotaxic frame (Narishige Co., Tokyo, Japan). After exposure of the skull, a small hole was made in the scalp, 0.2 mm anterior and 5.5 mm lateral to the bregma, and the microinjection needle was inserted through the hole to a depth of 4.5 mm (Par2 region). [<sup>3</sup>H]PAH (0.125 μCi/rat) or [<sup>3</sup>H]PCG (0.125 μCi/rat) with [<sup>14</sup>C]carboxyl-inulin (2.5 nCi/rat), which is a non-permeable reference compound, dissolved in 0.5 μl ECF buffer (122 mM NaCl, 25 mM NaHCO<sub>3</sub>, 10 mM D-glucose, 3mM KCl, 1.4 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 0.4 mM K<sub>2</sub>HPO<sub>4</sub>, and 10 mM HEPES, pH 7.4) in the presence or the absence of different concentrations of various inhibitors was injected into the Par2 region. The actual concentrations of labeled compounds in the injectate were 55 μM, 13 μM, and approximately 380 μM for [<sup>3</sup>H]PAH, [<sup>3</sup>H]PCG, and [<sup>14</sup>C]carboxyl-inulin, respectively. After intracerebral injection of the test compounds, rats were decapitated and the left and right cerebrum were removed at appropriate times. The excised cerebrum was dissolved in 2.5 ml 2N NaOH at 55 °C overnight, and the radioactivity in the brain specimens was measured in a liquid scintillation counter (LS 6000SE; Beckman Instruments., Fullerton, CA) after adding 14 ml scintillation fluid (Hionic-fluor; Packard Instruments, Meriden, CT). The 100-BEI (%) which represents the remaining percentage of the test compounds in the cerebrum is described by the following equation.

$$100 - \text{BEI} (\%) = \frac{\frac{\text{Amount of test drug in the brain}}{\text{Amount of reference in the brain}}}{\frac{\text{Amount of test drug injected}}{\text{Amount of reference injected}}} \times 100$$

The elimination rate constant of the compounds from the brain ( $k_{el}$ ) was obtained by

fitting the 100-BEI(%) versus time data. A nonlinear least-squares regression program (MULTI) (Yamaoka et al., 1981) was used for the calculation.

## Results

### The Expression of rOat3 in Brain Capillaries

The expression of rOat1, rOat2, and rOat3 in brain capillaries was examined using RT-PCR analysis (Fig. 1A). Amplified products of the expected size (499 bp) were detected in the brain capillary-enriched fraction as well as in the kidney, which is a positive control, using specific primer sets for rOat3. No amplified products were detected in the brain capillary-enriched fraction using specific primer sets for rOat1 and rOat2, although PCR products of the expected size (534 bp for rOat1 and 409 bp for rOat2) were detected in rat kidney and liver, respectively. The quality of the cDNA was confirmed by the amplification of  $\beta$ -actin fragments. DNA sequencing of the amplified products was identical to rOat1, rOat2, and rOat3, respectively.

The expression of rOat3 in brain capillaries was also confirmed at the protein level by Western blot analysis (Fig. 1B). An antiserum against rOat3 recognized approximately 75- and 65-kDa proteins in the brain capillary-enriched fraction and kidney plasma membrane fraction, respectively (lane 1, 2). These bands were abolished when preabsorbed antiserum for rOat3 was used (lane 3, 4), suggesting that the positive bands were specific for the antigen peptide.

To examine the degree of glycosylation of rOat3 in the brain capillary-enriched fraction and kidney, protein samples were deglycosylated with *N*-glycosidase F and subsequently subjected to Western blot analysis (Fig 1C). The resulting bands show the same molecular weight for the brain capillary-enriched fraction and kidney plasma membrane fraction (lane 3, 4), suggesting that the difference in the molecular weight is due to the difference in the degree of glycosylation between the brain capillaries and the

kidney.

### **Immunohistochemical Staining of rOat3**

The localization of rOat3 at the BBB was examined by immunohistochemical staining using frozen sections of rat brain. Positive staining signals were observed around the brain microvessels for rOat3 (green) and P-gp (red) (Fig. 2A), and the intensity of each signal was plotted along a cross-section of the microvessel (Fig. 2B). rOat3 produced a wider signal across the microvessel than P-gp, which is a luminal marker protein (Tsuji et al., 1992). Furthermore, the signal for rOat3 (green) did not overlap with that for GFAP (red), a marker for astrocytes, indicating that rOat3 is not expressed in the astrocytes but in the BCEC (Fig. 2C). These profiles strongly suggest that rOat3 is located on the abluminal membrane of the BCEC. The signal for rOat3 overlapped that for P-gp to some extent, indicating that rOat3 may be localized on the luminal as well as the abluminal membrane of the BCEC. Such characteristic immunostaining did not appear when normal rabbit serum was used as a negative control (Fig. 2D, red: P-gp).

### **Time Profile of the Efflux of [<sup>3</sup>H]PAH and [<sup>3</sup>H]PCG from the Brain across the BBB**

The time profile of the remaining percentage of PAH or PCG in the cerebrum after intracerebral injection is shown in Figure 3. Approximately 40% and 60% of the administered dose of PAH and PCG was eliminated from the cerebrum into the systemic circulation within 20 min, respectively. The apparent elimination rate constant ( $k_{el}$ ) was determined as  $0.039 \pm 0.004 \text{ min}^{-1}$  for PAH and  $0.043 \pm 0.006 \text{ min}^{-1}$  for PCG.

### **Concentration-dependent Efflux of PAH and PCG from the Brain and Mutual Inhibitory Effects**

The apparent elimination rate constant ( $k_{el}$ ) of PAH or PCG was reduced in a

dose-dependent manner (Fig. 4A, B). Taking the dilution factor of 45 in the cerebrum into consideration (Kakee et al., 1996), the Michaelis-Menten constant,  $K_m$ , for the efflux of PAH and PCG across the BBB was  $168 \pm 84 \mu\text{M}$  and  $28.7 \pm 4.5 \mu\text{M}$ , respectively (TABLE 1).

The efflux of [ $^3\text{H}$ ]PAH or [ $^3\text{H}$ ]PCG from the brain was inhibited by unlabeled PCG or unlabeled PAH, respectively, in a concentration-dependent manner (Fig. 4C, D). According to the kinetic analyses, the  $K_i$  values of PAH and PCG for the elimination of PCG and PAH from the cerebrum across the BBB were  $106 \pm 36 \mu\text{M}$  and  $58.1 \pm 15.8 \mu\text{M}$ , respectively (TABLE 1).

### **Effect of Cimetidine and Pravastatin on the Efflux of [ $^3\text{H}$ ]PAH and [ $^3\text{H}$ ]PCG from the Brain Parenchyma**

Cimetidine, which is not only a typical substrate of the Organic Cation Transporter (OCT) family (Dresser et al., 2001) but also a substrate of rOat3 (Nagata et al., 2002), and pravastatin, which is a substrate of rOat3 (Hasegawa et al., 2002), were used as inhibitors to confirm the involvement of rOat3 in the elimination of [ $^3\text{H}$ ]PAH and [ $^3\text{H}$ ]PCG from the cerebrum. As shown in Figure 5A and 5B, the inhibitory effects of cimetidine and pravastatin were dose-dependent and statistically significant.

## Discussion

In the present study, the expression of rOat3 in the BCEC was demonstrated by RT-PCR analysis, Western blotting, and immunohistochemical staining. In the RT-PCR analysis (Fig. 1A), the amplified product of rOat3 was detected in the brain capillary-enriched fraction as well as in the kidney, which corresponds to a previous report (Ohtsuki et al., 2002). In contrast, no expression of rOat1 or rOat2 at the BBB was detected in this study. Therefore, we conclude that, among the rat Oat family, only rOat3 is expressed at the BBB. The existence of rOat3 in the BCEC was further confirmed by Western blot analysis (Fig. 1B). The molecular weight of the positive signal detected in the brain capillary-enriched fraction was greater than that in the kidney plasma membrane fraction. This difference in the molecular weight was shown to be due to the difference in the degree of glycosylation between the brain capillaries and kidney (Fig. 1C). There is one additional band around 48 kDa in the brain capillary-enriched fraction. This may represent a degradation product of rOat3 arising during the isolation of the brain capillaries, since this band was abolished when preabsorbed antiserum for rOat3 was used. Immunohistochemical staining demonstrated that rOat3 is located on the abluminal membrane of the BCEC (Fig. 2). It is possible that rOat3 is expressed on the luminal as well as the abluminal membrane of the BCEC, since the signal for rOat3 overlapped that for P-gp to some extent.

Intracerebrally administered PAH and PCG were eliminated in a saturable manner with rate constants of  $0.039 \text{ min}^{-1}$  and  $0.043 \text{ min}^{-1}$ , respectively (Figs. 3 and 4). The efflux rate of PAH estimated in the present study was close to the reported value of  $0.059 \text{ min}^{-1}$  (Kakee et al., 1997). The  $K_m$  values of the elimination of PAH and PCG from the



cerebrum across the BBB were 168  $\mu\text{M}$  and 29  $\mu\text{M}$ , respectively. The  $K_m$  value of the efflux of PAH was similar to the reported value of 267  $\mu\text{M}$ , taking the dilution factor of 45 in the cerebrum into account (Kakee et al., 1997). The efflux of PCG and PAH was inhibited in a dose-dependent manner by PAH and PCG with  $K_i$  values of 106  $\mu\text{M}$  and 58  $\mu\text{M}$ , respectively. These values are comparable with their  $K_m$  values, indicating that PAH and PCG share the same efflux transport system at the BBB. Furthermore, this was supported by the inhibition study using cimetidine and pravastatin as inhibitors (Fig. 5). The degree of inhibition of the elimination from the cerebrum by cimetidine and pravastatin was similar for PAH and PCG. Considering that PAH and PCG are substrates of rOat3 and that rOat3 is expressed on the abluminal membrane of the BCEC, the  $K_m$  and  $K_i$  values determined in this study may reflect the affinity of PAH and PCG for rOat3. Indeed, these values were comparable with the  $K_i$  and  $K_m$  values of PAH and PCG (398  $\mu\text{M}$  for PAH and 82.6  $\mu\text{M}$  for PCG) for the rOat3 expression system reported previously (Nagata et al., 2002). The inhibitory effect of cimetidine and pravastatin, substrates of Oat3, on the elimination of PAH and PCG from the brain across the BBB further supports the participation of rOat3 in this process. The  $K_i$  and  $K_m$  values of cimetidine and pravastatin for rOat3 are 47  $\mu\text{M}$  and 13  $\mu\text{M}$ , respectively (Hasegawa et al., 2002; Nagata et al., 2002), suggesting that the degree of inhibition by these inhibitors observed here may be reasonable, although further studies are required to determine their *in vivo*  $K_m$  and  $K_i$  values. These results support our speculation that rOat3 is involved in the elimination of PAH and PCG from the brain across the BBB.

The apparent elimination rate constants of PAH and PCG were similar (Fig. 3), although the intrinsic transport activity of PCG by rOat3 was much greater than that of

PAH (Kusuhara et al., 1999; Nagata et al., 2002). There are two possibilities to account for this discrepancy. The first is a difference in the distribution volume in the brain. The elimination rate constant is obtained from the efflux clearance, an intrinsic parameter for the efflux transport activity across the BBB, divided by the distribution volume in the brain (Kakee et al., 1996). If the distribution volume of PCG is much greater than that of PAH, it is possible that the elimination rate constants of PAH and PCG are comparable even though the efflux transport activity of PCG is greater than that of PAH. The second is a difference in the rate of luminal secretion. The elimination of compounds from the cerebrum into the systemic circulation consists of two steps, i.e. uptake across the abluminal membrane and subsequent excretion across the luminal membrane. The apparent elimination rate constant determined by the BEI method represents a combination of these two processes. Assuming that the rate-limiting step of the efflux of PCG from the brain across the BBB is luminal excretion and the rate is smaller than that of PAH, similar apparent elimination rate constants for PAH and PCG will be observed.

A carrier-mediated influx of PCG from the blood to the brain via the BBB has been shown by an *in situ* brain perfusion technique (Suzuki et al., 1989). The major transport system of PCG from the blood to the brain via the BBB is by a saturable process with a  $K_m$  value of approximately 8-30  $\mu\text{M}$ . Weak immunofluorescence signals for rOat3 were detected along the luminal membrane of the brain capillaries (Fig. 2). In addition, the  $K_m$  value of the brain uptake of PCG was comparable with that of the efflux of PCG from the cerebrum across the BBB, i.e. 29  $\mu\text{M}$ , determined in this study and that for the rOat3 expression system, i.e. 82.6  $\mu\text{M}$ , reported previously (Nagata et al., 2002). Thus, it is possible that rOat3 can account for the uptake of PCG from the blood to the brain via the

BBB. This possibility should be examined using inhibitors of rOat3 and/or Oat3 knockout mice (Sweet et al., 2002b) to confirm the functional involvement of rOat3 in the uptake of its substrates from the circulating blood. Furthermore, it has been reported that rOat3 functions as an organic anion/dicarboxylate exchanger like rOat1 (Sweet et al., 2002a). It is speculated that rOat3 can mediate both uptake and efflux across the plasma membrane depending on the concentration gradient of the substrate and its driving force, and that rOat3 is involved in the excretion across the luminal membrane of the brain capillaries. Another candidate among the efflux transporters for organic anions on the luminal membrane may be multidrug resistance associated protein 2 (Mrp2), since the expression of this transporter on the luminal membrane of the BCEC has been reported (Miller et al., 2000) and ATP-dependent accumulation of PAH into membrane vesicles expressing Mrp2 has also been reported (Van Aubel et al., 2000). Mrp1 as well as Mrp2 would explain the efflux process, since this ABC transporter accepts PAH as a substrate (Leier et al., 2000), although the presence of Mrp1 at the BBB is debatable (Kusuhara and Sugiyama, 2001a; Kusuhara and Sugiyama, 2001b). Further studies are necessary to evaluate the contribution of the transporters to the total excretion across the luminal membrane of the brain capillaries.

A variety of compounds have been shown to be eliminated across the BBB in a carrier-mediated manner using the BEI method. These compounds include  $17\beta$ -estradiol-D- $17\beta$ -glucuronide ( $E_217\beta G$ ), estrone-3-sulfate ( $E_1S$ ), and indoxyl sulfate (IS) (Hosoya et al., 2000; Sugiyama et al., 2001; Ohtsuki et al., 2002). PAH inhibited the elimination of  $E_217\beta G$  and IS at a concentration of 300 mM and 10 mM in the injectate, respectively. However, 100 mM PAH in the injectate did not affect the elimination of

E<sub>1</sub>S, although this concentration is sufficient to saturate the efflux of PAH itself from the cerebrum. Since the degree of inhibition by PAH may reflect the contribution of rOat3, rOat3 appears to participate in the efflux of E<sub>2</sub>17βG and IS, although the contribution of rOat3 to the total efflux of E<sub>2</sub>17βG from the brain is approximately 20%. In contrast, although E<sub>1</sub>S is reported to be transported by rOat3 (Kusuhara et al., 1999), PAH had no effect on the efflux of this compound in the *in vivo* situation. This discrepancy may be accounted for by the minor contribution of rOat3 to the efflux of E<sub>1</sub>S from the brain across the BBB.

The physiological role of Oat3 has been discussed (Kusuhara et al., 1999; Ohtsuki et al., 2002). Since the BCEC are connected to each other by highly developed tight junctions, it is unlikely that hydrophilic compounds such as metabolites of neurotransmitters are transported through the BBB via paracellular passive diffusion. Thus, it seems that there are efflux transporters at the BBB able to pump some hydrophilic substrates out of the brain. It has been shown that metabolites of neurotransmitters such as dopamine, norepinephrine, epinephrine, serotonin, and histamine are inhibitors for rOat3, suggesting that these compounds are endogenous substrates of Oat3. rOat3 may be involved in the elimination of endogenous waste material as well as exogenous hydrophilic organic anions from the brain to maintain brain homeostasis. The expression of rOat3 in the BCEC and choroid plexus, the interface between the brain interstitial fluid or CSF and the blood circulation, supports this hypothesis. Further studies, including demonstration of the dose-dependent elimination of hydrophilic metabolites from the brain into the circulation across the BBB and BCSFB, will be required to identify the precise physiological function of Oat3 in the

brain.

The human homologue of rOat3, hOAT3, was isolated from a human kidney cDNA library (Cha et al., 2001). The substrate specificity of hOAT3 is similar to that of rOat3 and Northern blot analysis revealed that hOAT3 mRNA is expressed in the kidney and, to the lesser extent, in the brain and skeletal muscle. It is possible that hOAT3 is localized at the BBB and BCSFB in the brain and is involved in regulating the concentrations of endogenous and exogenous substrates in the central nervous system, considering the characteristics of rOat3 in the brain reported by us and other groups (Ohtsuki et al., 2002).

In conclusion, rOat3 is the dominant isoform of the Oat family expressed in the BCEC and plays an important role in the elimination of PAH and PCG from the cerebrum into the systemic circulation across the BBB.

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### Legends to figures

#### **Figure 1. RT-PCR analysis (A), Western blot analysis (B), and Deglycosylation of**

**rOat3 (C).** (A) Total RNA from rat brain capillary-enriched fraction (B), kidney (K), and liver (L), or distilled water (W) was subjected to RT-PCR analysis using primer sets specific for the rOat1, rOat2, rOat3, and  $\beta$ -actin gene. The identity of the PCR products is shown on the left. (B) Isolated rat brain capillaries (10  $\mu$ g, lane 1) and kidney plasma membrane (1  $\mu$ g, lane 2) were separated by SDS-PAGE (10% separating gel). rOat3 was detected by horseradish peroxidase-labeled anti-rabbit IgG after incubation with antiserum raised against rOat3. Immunoreactivity was completely abolished by pretreatment of antibody with the antigen (lane 3, 4). B, brain capillary-enriched fraction; K, kidney plasma membrane (C) Rat brain capillary proteins (10  $\mu$ g, lane 1, 3) and kidney plasma membrane proteins (3  $\mu$ g, lane 2, 4) were treated with (lane 3, 4) or without (lane 1, 2) PNGase F and subsequently separated by SDS-PAGE (10% separating gel). rOat3 was detected as described in (B). B, brain capillary-enriched fraction; K, kidney plasma membrane

**Figure 2. Immunohistochemical staining.** (A) Cryosections of brain from male Sprague-Dawley rats were dual-stained with anti-rOat3 antiserum (green) and C219 (red). (B) Graphical analysis of fluorescence intensities along the cross-section a-b in (A). (C) Dual-staining with antibodies to rOat3 (green) and to GFAP (red). (D) Normal rabbit serum (green) was used as a negative control with C219 (red) for double-staining. Bars = 10  $\mu$ m.

**Figure 3. Time profile of [<sup>3</sup>H]PAH and [<sup>3</sup>H]PCG in the cerebrum after intracerebral microinjection.** A mixture of [<sup>3</sup>H]PAH (0.125 μCi/rat) or [<sup>3</sup>H]PCG (0.125 μCi/rat) and [<sup>14</sup>C]carboxyl-inulin (2.5 nCi/rat) dissolved in 0.5 μl ECF buffer was injected into Par2 of the rat cerebrum, and then animals were decapitated at appropriate times. Circles and squares represent the elimination of PAH and PCG, respectively. The solid line represents the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

**Figure 4. Concentration-dependence of the efflux of PAH and PCG across the BBB and their mutual inhibitory effects.** (A, D) Concentration-dependent efflux of [<sup>3</sup>H]PAH (A) and the effect of unlabeled PAH on the efflux of [<sup>3</sup>H]PCG (D). A mixture of [<sup>3</sup>H]PAH (0.125 μCi/rat) (A) or [<sup>3</sup>H]PCG (0.125 μCi/rat) (D) and [<sup>14</sup>C]carboxyl-inulin (2.5 nCi/rat) dissolved in 0.5 μl ECF buffer was injected into Par2 of the rat cerebrum in the presence of 0, 1, 10, 30, 100, 300 mM unlabeled PAH in the injectate. Rats were decapitated at 20 min after microinjection and the elimination rate constant ( $k_{el}$ ) was calculated. (B, C) Concentration-dependent efflux of [<sup>3</sup>H]PCG (B) and the effect of unlabeled PCG on the efflux of [<sup>3</sup>H]PAH (C). A mixture of [<sup>3</sup>H]PCG (0.125 μCi/rat) (B) or [<sup>3</sup>H]PAH (0.125 μCi/rat) (C) and [<sup>14</sup>C]carboxyl-inulin (2.5 nCi/rat) dissolved in 0.5 μl ECF buffer was administered in the presence of 0, 1, 3, 10, 30, 100 mM unlabeled PCG in the injectate and the elimination rate constant ( $k_{el}$ ) was calculated. (C, D) Results are given as a ratio with respect to the elimination rate constant determined in the absence of unlabeled inhibitors. (A through D) Solid lines represent the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

**Figure 5. Effects of unlabeled cimetidine and pravastatin on the efflux of [<sup>3</sup>H]PAH or [<sup>3</sup>H]PCG from the cerebrum.** ECF buffer containing [<sup>3</sup>H]PAH (A) or [<sup>3</sup>H]PCG (B) and [<sup>14</sup>C]carboxyl-inulin with or without unlabeled inhibitors was microinjected into Par2 of rat cerebrum and the elimination rate constant ( $k_{el}$ ) of [<sup>3</sup>H]PAH or [<sup>3</sup>H]PCG was determined. Each value of the expected concentration was estimated by the concentration in the injectate divided by the dilution factor of 45 (Kakee et al., 1996). Results are given as a ratio with respect to the elimination rate constant determined in the absence of unlabeled inhibitors. Each column represents the mean  $\pm$  S.E. (A; n = 4, B; n = 3). \* Significantly different from the control ( $p < 0.05$ ).

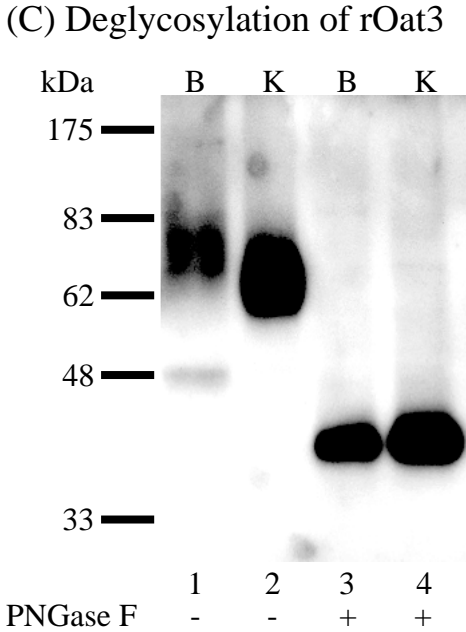
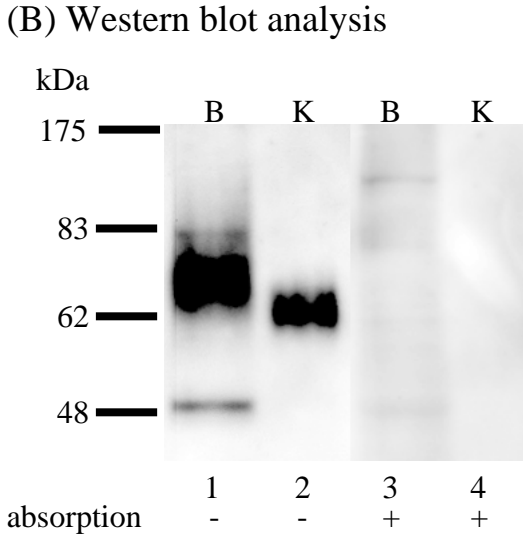
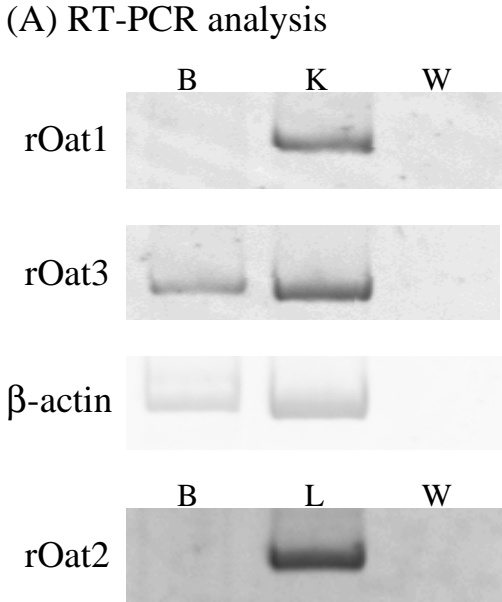
**TABLE1**

$K_m$  and  $K_i$  values for the efflux of PAH and PCG from the cerebrum across the BBB. The effect of unlabeled PAH or PCG on the efflux of [ $^3$ H]PAH and [ $^3$ H]PCG across the BBB was examined. These  $K_m$  and  $K_i$  values were determined by nonlinear regression analysis assuming competitive inhibition and 45-fold dilution in the cerebrum after microinjection. Data are taken from Figure 4. Values represent the mean  $\pm$  S.D. (n = 3).

	[ $^3$ H]PAH	[ $^3$ H]PCG
PAH	$K_m = 168 \pm 84 \mu\text{M}$	$K_i = 106 \pm 36 \mu\text{M}$
PCG	$K_i = 58.1 \pm 15.8 \mu\text{M}$	$K_m = 28.7 \pm 4.5 \mu\text{M}$

dilution factor = 45

Figure 1





# Figure 2

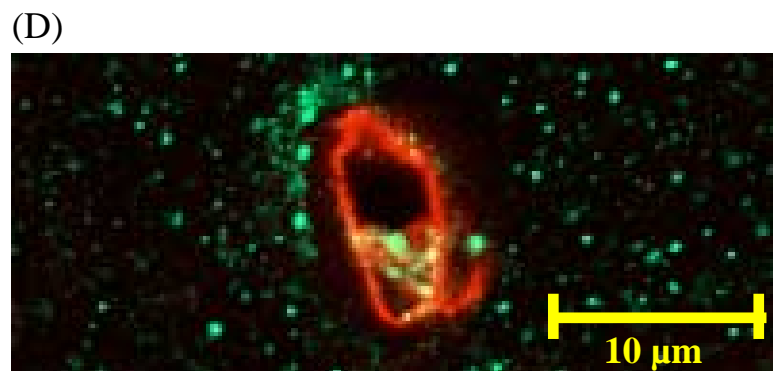
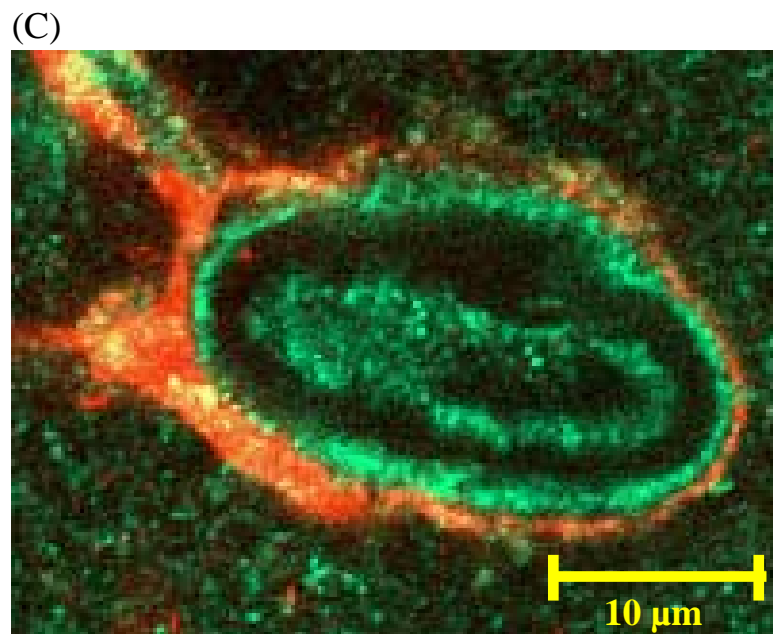
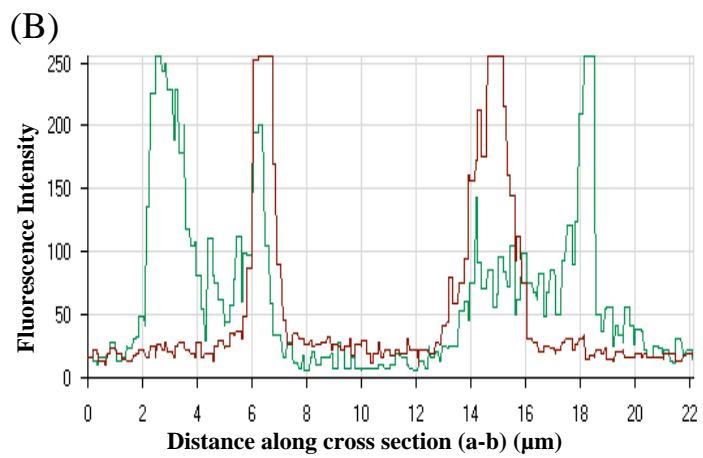
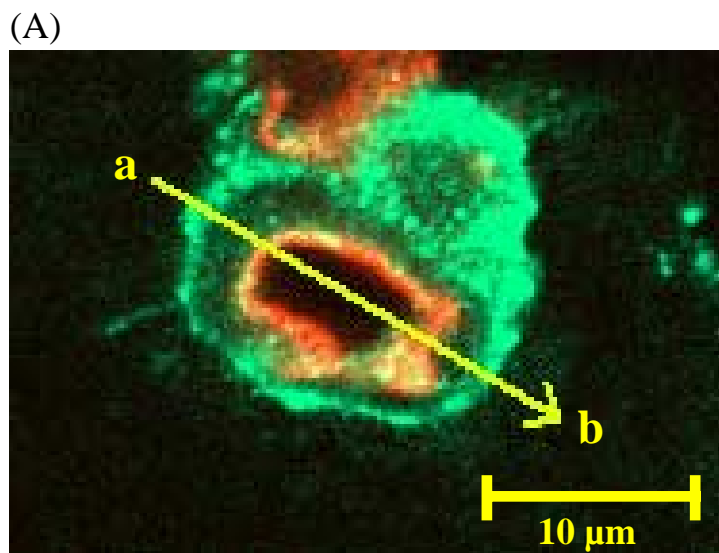


Figure 3

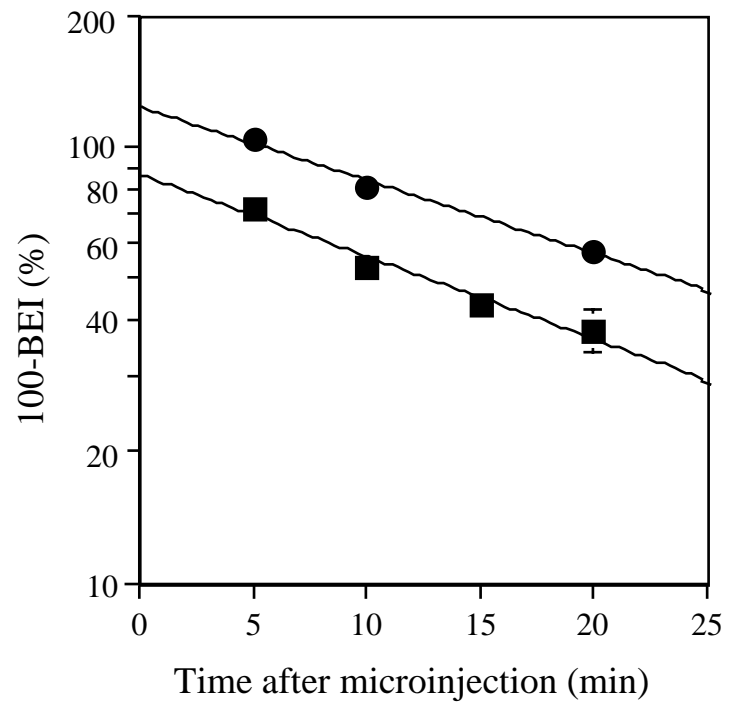
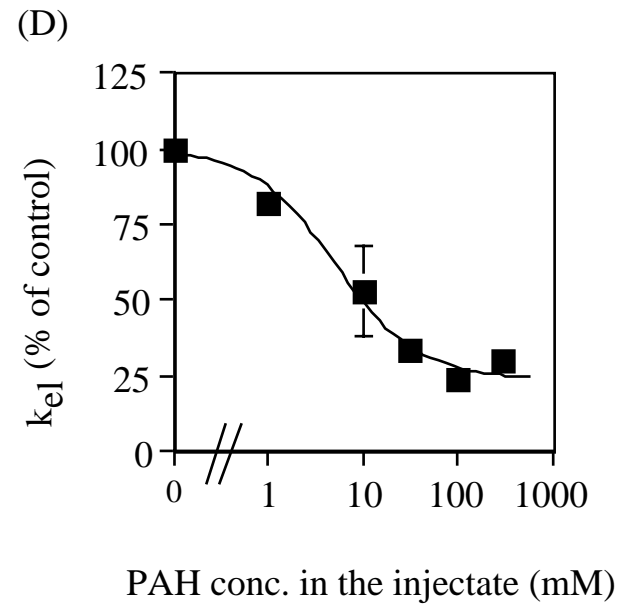
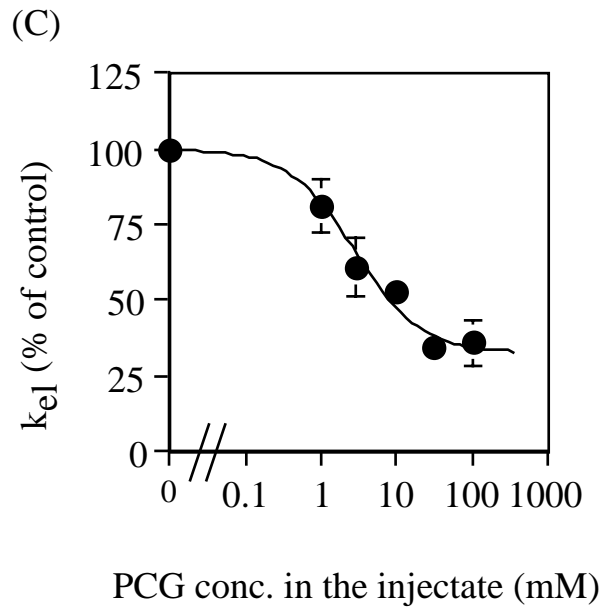
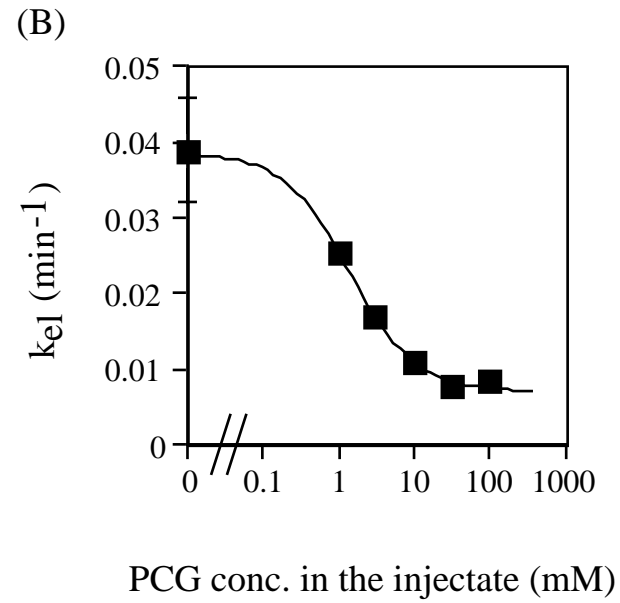
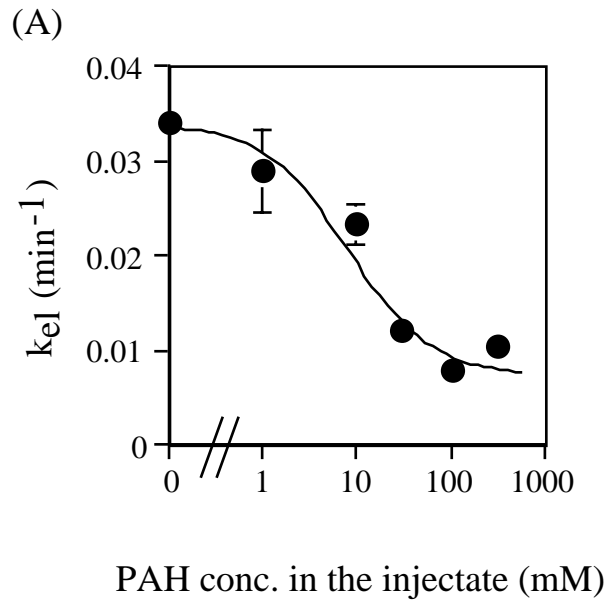


Figure 4



# Figure 5

