Characteristics of Choline Transport Across the Blood-Brain Barrier in Mice: Correlation with *In Vitro* Data

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Purpose. We examined the functional properties of choline transport across the blood-brain barrier (BBB) in mice. We compared the kinetic parameters and transport properties with those found in our *in vitro* uptake experiments using mouse brain capillary endothelial cells (MBEC4).

Methods. The permeability coefficient-surface area product (PS) values of [^{3}H]choline at the BBB were estimated by means of an *in situ* brain perfusion technique in mice.

Results. [^{3}H]Choline uptake was well described by a two-component model: a saturable component and a nonsaturable linear component. The [^{3}H]choline uptake was independent of pH and Na^+, but was significantly decreased by the replacement of Na^+ with K^+. Various basic drugs, including substrates and inhibitors of the organic cation transporter, significantly inhibited the [^{3}H]choline uptake. These *in situ* (*in vivo*) results corresponded well to the *in vitro* results and suggest that the choline transporter at the BBB is a member of the organic cation transporter (OCT) family.

Conclusion. The choline transport mechanism at the BBB is retained in MBEC4.

KEY WORDS: choline; *in situ* brain perfusion technique; blood-brain barrier; organic cation transporter; *in vivo* and *in vitro* correlation.

INTRODUCTION

Brain capillary endothelial cells are tightly bound to each other and constitute the blood-brain barrier (BBB), which serves to restrict the transport of compounds into the brain. Permeation of compounds across the BBB is determined not only by lipophilicity and molecular size, but also by various transporters on the endothelial cell membrane (1).

Choline is a precursor of acetylcholine as well as essential constituents of the cell membrane, such as phosphatidylcholine and sphingomyelin. Only small amounts of choline are synthesized in the brain and the rest, which is absorbed from the diet, must be transported into the brain (2–4). Therefore, transport of choline from blood to brain through the BBB is a physiologically important process. In *in vitro* uptake experiments in mouse brain capillary endothelial cells (MBEC4), we found that a membrane potential-dependent transporter contributes to choline transport across the BBB (5). However, transport mechanisms of cultured cells do not always reflect those *in vivo* at the BBB. In fact, MBEC4 does not express *mdr1a* gene product, but expresses *mdr1b* gene product, although only *mdr1a* gene product was detected in mouse BBB *in vivo* (6). Therefore, it is important to confirm that the choline transporters operating at the BBB *in vivo* and in MBEC4 *in vitro* are identical.

The purpose of the present study was to clarify the functional properties of choline transport across the BBB and to investigate the effects of various basic drugs on the transport by means of an *in situ* brain perfusion technique in mice (7). In addition, we confirmed that the choline transporter in MBEC4 is identical to that in the BBB by comparing the kinetic parameters and transport properties found here with our previous *in vitro* data for MBEC4 (5).

MATERIALS AND METHODS

Materials and Animals

[^{3}H]Choline chloride (specific activity, 83.0 Ci/mmol) was purchased from Amersham International plc (Buckinghamshire, England). [^{14}C]Sucrose (specific activity, 495 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (CA). Choline chloride, hemicholinium-3 (HC-3) and *N*-methylnicotinamide (NMN) were from Aldrich Chemical Company, Inc. (Milwaukee, WI). Procainamide hydrochloride was from Sigma Chemical Co. (St. Louis, MO). Quinine sulfate, tetraethylammonium bromide (TEA), guanidine hydrochloride, *L*-arginine, *L*-lysine monohydrochloride, *p*-aminohippuric acid (PAH), acetylcholine chloride, dopamine chloride, 5-hydroxytryptamine hydrochloride (serotonin) and histamine dihydrochloride were from Nacalai Tesque, Inc. (Kyoto, Japan). Bunitrolol was kindly supplied by Boehringer Ingelheim (Ingelheim am Rhein, Germany). Solvable was obtained from Packard Instruments (Downers Grove, IL). All other chemicals were commercial products of reagent grade. The male ddY mice (20–25 g) were obtained from Seac Yoshitomi, Ltd. (Fukuoka, Japan).

Surgical Procedure

Surgery was performed according to our previous report (7). In brief, adult male mice (20–25 g) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) (Dainippon Pharmaceutical, Osaka, Japan). The common carotid artery was exposed, the occipital and superior thyroid arteries were coagulated and cut, and the pterygopalatine artery was ligated. The external carotid artery was catheterized for retrograde infusion with polyethylene tubing. The carotid artery was prepared for ligation by encircling it with surgical thread.

Perfusion Fluid

Krebs-Henseleit buffer consists of 118.0 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 2.5 mM $CaCl_2$. Prior to an experiment, 10 mM D-glucose was added and the perfusate was oxygenated for 10 min with 95% O_2-5% CO_2, and adjusted to pH 7.4 with HCl. In the study of concentration-dependency, choline concentration of the perfusate was adjusted by adding unlabeled choline. For the investigation of the effect of pH, the perfusate

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was adjusted to pH 8.0 or 6.0 with HCl or mesylate (MES), respectively. In the ion replacement study, NaCl in the perfusate was replaced by *N*-methylglucamine or KCl, and $NaHCO_3$ was replaced by a stoichiometrically equivalent amount of $KHCO_3$. In addition, the effect of 10 μM valinomycin was also studied. In the inhibition study, procainamide, TEA, NMN, guanidine, HC-3, quinine, acetylcholine, dopamine, histamine, serotonin, *L*-arginine, *L*-lysine, or PAH was dissolved in the perfusate to give a final concentration of 10 mM except for quinine, which was used at 1 mM because of its low solubility. All of the perfusates also contained 5 nM [^{3}H]choline and were equilibrated at 37°C. In addition, 0.2 μM [^{14}C]sucrose was added to estimate intravascular volume.

Perfusion System

The perfusate was infused into the external carotid artery at a constant rate by an infusion pump (model 55-1111, Harvard Apparatus, Holliston, MA) and the common carotid artery was ligated immediately. The perfusion rate was 1.0 ml/min and perfusion was continued for 15, 30, or 60 s, then terminated by decapitation. The cerebral hemispheres were immediately excised, weighed, and placed in a scintillation vial. Samples were digested in 1.5 ml of tissue solubilizer, neutralized by addition of 100 μl of 6 M HCl, and prepared for scintillation counting by addition of 15 ml of scintillation cocktail. The radioactivity of the brain or perfusate samples was measured with a liquid scintillation counter (model LS6500, Beckman Instruments, Inc., Fullerton, CA). It was confirmed that the BBB remained intact during the study, because the intravascular volume estimated from the distribution volume of [^{14}C]sucrose was constant (11–14 μl/g brain).

Calculation

Brain uptake of drug during the perfusion is given by Eq. (1):

$$dC_{brain}/dt = K_1 \times C_{pf} \quad (1)$$

where C_{brain} is the brain concentration of tracer (pmol/g brain), C_{pf} is the concentration of tracer in the perfusate (pmol/ml), and K_1 is influx clearance (ml/g brain/s). Because C_{pf} is constant, we may divide Eq. (1) by C_{pf} to obtain Eq. (2),

$$dK_p/dt = K_1 \quad (2)$$

where K_p (ml/g brain) is the ratio of C_{brain} to C_{pf}. As shown in Fig. 1, K_p is linearly related to K_1, so we obtain Eq. (3):

$$K_1 = K_p/t \quad (3)$$

The permeability coefficient-surface area product (PS) values at the BBB were calculated from Eq. (4),

$$PS = -F_{pt} \times \ln(1 - K_p/F_{pf}/t)$$

where F_{pf} is cerebral perfusion fluid flow. F_{pf} calculated from brain uptake data of [^{14}C]diazepam for 5 s was 71.3 μl/g brain/s (7).

Uptake rate (J; pmol/g brain/s) is given by Eq. (5):

$$J = PS \times C_{pf} \quad (5)$$

To estimate the kinetic parameters, the uptake rate was fitted to Eq. (6), which contains both saturable and nonsaturable linear terms, using the nonlinear least-squares regression analysis program, MULTI (8):

$$J = J_{max} \times C/(K_t + C) + k_d \times C \quad (6)$$

where J_{max} is the maximum uptake rate for the saturable component (pmol/g brain/s, K_t is the Michaelis constant (μM), k_d is the first-order constant for the nonsaturable component (μl/g brain/s), and C is the concentration of choline (μM).

Data are expressed as mean ± SEM. The significance of differences was evaluated by ANOVA followed by Duncan's test.

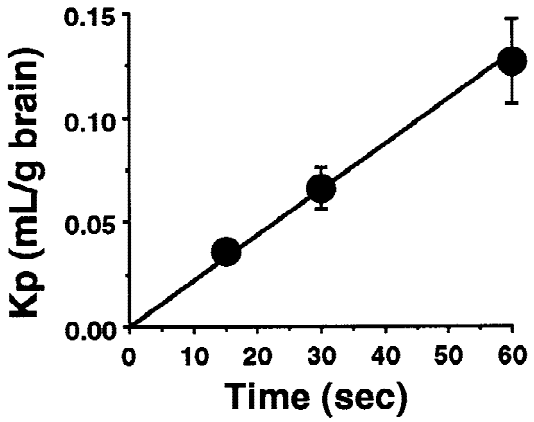

Fig. 1. K_p value-time profile of [^{3}H]choline transport across the BBB. The line shows the simulation using Eq. (3). Each point represents the mean ± SEM for three experiments.

Scale-up from *In Vitro* Data to *In Vivo*

To predict transport rates in mouse whole brain, we corrected kinetic parameters estimated in *in vitro* uptake experiments (5) by using cultured area per protein amount (10 cm^2/mg protein; our experimental data) and surface area of brain capillary endothelial cells per weight of brain (100 cm^2/g brain) (9), and we compared the values obtained with the *in vivo* kinetic parameters obtained in this study.

RESULTS

Time Course of [^{3}H]Choline Uptake

Figure 1 shows the K_p value-time profile of [^{3}H]choline. The relationship was linear for up to 60 s. We chose perfusion time of 30 s for all subsequent experiments.

Concentration Dependency of [^{3}H]Choline Uptake

As shown in Fig. 2, the initial uptake rate of choline was saturated as the concentration increased. Using Eq. (6), J_{max}, K_t, and k_d were estimated to be 74.6 ± 23.3 pmol/g brain/s, 41.3 ± 13.0 μM, and 0.26 ± 0.14 μL/g brain/s, respectively. These results suggest that carrier-mediated transport is involved in choline distribution into the brain.

Effect of pH on [^{3}H]Choline Uptake

The uptake of [^{3}H]choline into the brain was not affected by pH. The PS values of [^{3}H]choline at pH 6.0, 7.4, and 8.0

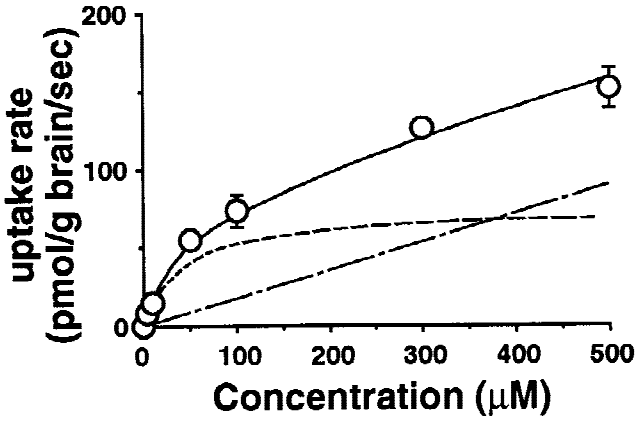

Fig. 2. Concentration dependence of choline uptake across the BBB. Initial uptake rates at various concentrations of choline (5 nM–500 μM) were measured at 37°C for 30 s. Each point represents the mean ± SEM for three experiments. Curves for total uptake (solid line), the saturable component (dotted line), and the nonsaturable component (broken line) were calculated using the parameters obtained.

were 1.44 ± 0.11, 1.50 ± 0.10, and 1.32 ± 0.13 μL/g brain/s (mean ± SEM, $n = 3$), respectively.

Effects of Ionic Composition of the Perfusate on [^{3}H]Choline Uptake

Experiments were performed with various ionic compositions of perfusate. Figure 3 shows PS values of [^{3}H]choline after the replacement of Na^+ with *N*-methylglucamine$^+$ or K^+. The PS value of [^{3}H]choline was not affected by the replacement with *N*-methylglucamine$^+$, whereas it was significantly decreased by the replacement with K^+. Addition of 10 μM valinomycin, an ionophore of K^+, did not afford any further decrease in [^{3}H]choline uptake.

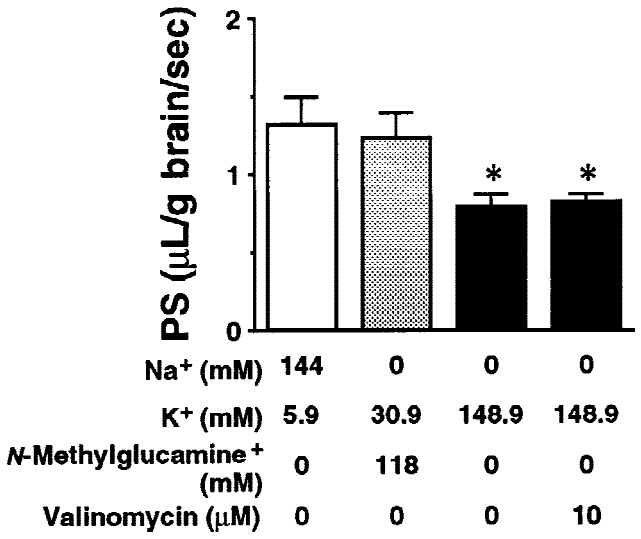

Fig. 3. Effects of perfusate ionic composition on [^{3}H]choline transport across the BBB. The PS value of [^{3}H]choline transport was measured for 30 s in normal perfusate (control; open column) or perfusate in which Na^+ was replaced by *N*-methylglucamine$^+$ (dotted column) or K^+ (solid column) and which contained valinomycin (10 μM). Each result represents the mean ± SEM for three experiments. The significance of differences from the control was determined by ANOVA followed by Duncan's test (*$p < 0.05$).

Effects of Basic Drugs on [^{3}H]Choline Uptake

The inhibitory effects of various basic drugs on [^{3}H]choline uptake are shown in Fig. 4. The PS value of [^{3}H]choline was significantly decreased by TEA, procainamide, quinine, bunitrolol, and HC-3, and somewhat inhibited by NMN and guanidine. In addition, neurotransmitters such as acetylcholine, dopamine, histamine, and serotonin significantly reduced the uptake of [^{3}H]choline. On the other hand, basic amino acids (*L*-lysine and *L*-arginine) and an organic anion (PAH) had no effect.

DISCUSSION

Properties of Choline Transport Across the BBB in Mice

First, using the *in situ* brain perfusion technique, we investigated the kinetic properties of choline transport into the brain in mice. Cornford *et al.* reported carrier-mediated transport of choline across the BBB in rats (4). However, our *in situ* perfusion study has the advantages of excluding the effects of plasma protein binding or peripheral metabolism, and constant concentrations of substrate and inhibitor can be maintained throughout the experiments.

The PS value of [^{3}H]choline was significantly decreased

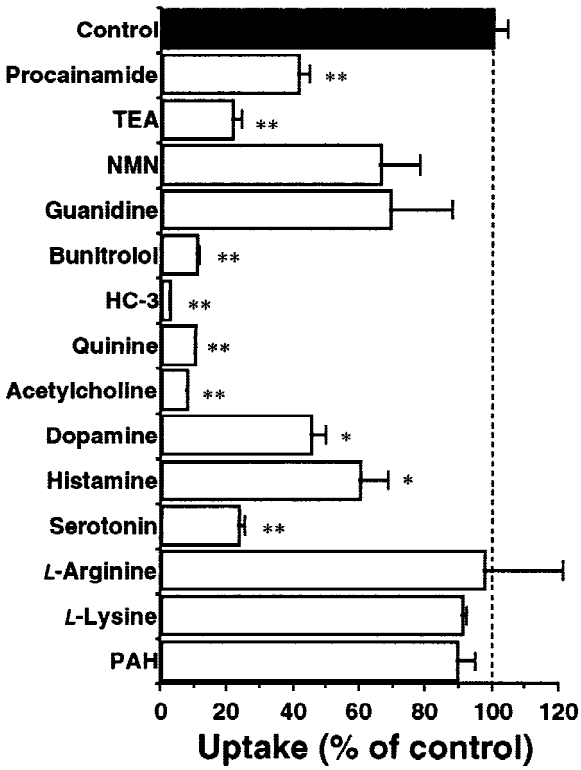

Fig. 4. Effects of various drugs on [^{3}H]choline transport across the BBB. The PS value of [^{3}H]choline transport was measured for 30 s in the presence (open column) or absence (control; solid column) of 10 mM inhibitor (except for quinine; 1 mM). Each result represents the mean ± SEM for three experiments. The significance of differences from the control was determined by ANOVA followed by Duncan's test (*$p < 0.01$, **$p < 0.001$).

by the replacement of Na^+ with K^+. This finding suggests that transport of choline is membrane potential-dependent. However, [^{3}H]choline uptake was not affected by further addition of valinomycin. Because approximately 60% of choline transport was not affected, involvement of another active transport system cannot be excluded. However, the most plausible explanation for this is that the perfusion period was too short for the effect of K^+ and valinomycin to appear. The PS value of [^{3}H]choline was not affected by replacement of Na^+ with *N*-methylglucamine$^+$ or by change of the pH. These findings indicate that the transport of choline across the BBB is independent of sodium ion or pH.

In inhibition experiments, we demonstrated that the choline transporter recognizes basic drugs or neurotransmitters, as well as choline (Fig. 4), suggesting that it may contribute to the brain distribution of these substances. Recently, the dopamine transporter (DAT) and serotonin transporter (SERT) were identified in the ends of synapses and were cloned (10,11). These transporters, which are dependent on sodium and chloride and have lower K_t values (890 nM for DAT, 320 nM for SERT), may not contribute to the transport of choline across the BBB (12).

The plasma choline concentration in humans or rats is approximately 10 μM (13–15). Assuming the same concentration of choline in mice, the choline transporter identified in this study should contribute predominantly to the choline transport under physiological conditions, since its K_t value is 40 μM.

Functional Similarity Between OCT Family and the Choline Transporter

Recently, an organic cation transporter (OCT1) located on the basolateral membrane of renal proximal tubules has been cloned (16). Subsequently, other homologues including OCT2, OCT3, OCTN1, OCTN2, and OCTN3 have been reported (17–21), and collectively they have been designated as the organic cation transporter (OCT) family. The choline transporter described here appears to be a member of the OCT family. First, the choline transporter is membrane potential-dependent, like OCT1, OCT2, and OCT3 (16–18). Second, uptake of choline in this study was inhibited by substrates or inhibitors of OCT family members, i.e., TEA, procainamide, and quinine. It should be mentioned that neurotransmitters such as acetylcholine, dopamine, histamine, and serotonin are known to be transported by OCT1 and OCT2 (22–24). Third, OCT1 was reported to transport choline (25). With other members of the OCT family, transport of substrates was inhibited by choline, suggesting that choline is also a substrate of these transporters (26–29). Therefore, it is strongly suggested that the choline transporter at the BBB found in this study is indeed one (or more) of the isoforms of the OCT family, or an unknown homologue.

In Vivo-In Vitro Correlation of Choline Transport

We compared kinetics parameters and functional properties of choline transport between *in vivo* and *in vitro* (5). To scale-up to *in vivo* mouse whole brain, we corrected the kinetic parameters estimated by *in vitro* uptake experiments for both cultured area per protein amount (10 cm^2/mg protein; our experimental data) and surface area of brain capillary endothelial cells per weight of brain (100 cm^2/g brain) (9). As shown in Table 1, good agreement was found, with K_t values of 41 and 20 μM, J_{max} of 74.6 and 70.5 pmol/g brain/s, k_d of 0.26 and 0.21 μl/g brain/s, and K_1 ($= J_{max}/K_t$) of 2.10 and 3.51 μl/g brain/s *in vivo* and *in vitro,* respectively. Functional properties such as membrane potential-dependence and sodium ion- and pH-independence in this study were also in good correspondence with those *in vitro.* Moreover, as shown in Fig. 5, the inhibitory potencies (percent of control) for most drugs lie near the 1:1 line, showing a good correspondence between *in vivo* and *in vitro* behavior. However, in the case of guanidine, a marked difference of its inhibitory effects *in vitro* and *in situ* was observed. Expression of a guanidine-specific transporter on the brush border membrane of the small intestine has been reported (30). A possible explanation for the

Table 1. Comparison of Choline Transport In Vivo and In Vitro

	In situ mouse brain perfusion technique	In vitro uptake experiment by MBEC4 cells[a]
Michaelis constant (K_t; μM)	41.3 ± 13	20.0 ± 3.1
Maximum flux (J_{max}; pmol/g brain/s)	74.6	70.5
Transfer constant for diffusion (k_d; μl/g brain/s)	0.26	0.21
Influx clearance (K_1 or J_{max}/K_t; μl/g brain/s)	2.10	3.51
Membrane potential-dependent	+	+
pH-dependent	–	–
Na-dependent	–	–

[a] Data from our previous report (reference 5).

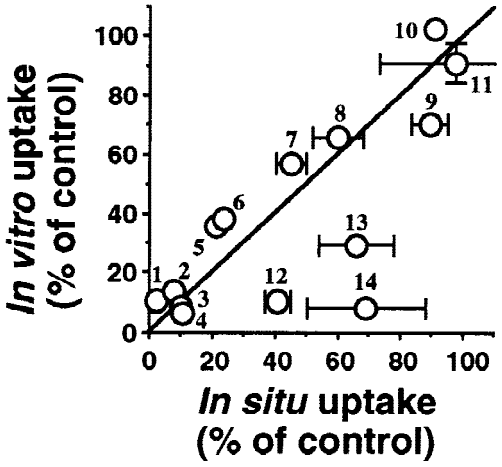

Fig. 5. Comparison of inhibitory effects of various drugs on [^{3}H]choline uptake between *in vitro* and *in situ*. Inhibitory effects of various drugs (1) HC-3; (2) acetylcholine; (3) quinine; (4) bunitrolol; (5) procainamide; (6) serotonin; (7) dopamine; (8) histamine; (9) PAH; (10) *L*-lysine; (11) *L*-arginine; (12) TEA; (13) NMN; (14) guanidine) on [^{3}H]choline uptake were estimated previously in an *in vitro* uptake experiment or by the *in situ* brain perfusion technique. Each point represents the mean ± SEM for three (*in situ*) or four (*in vitro*) experiments. The line is the line of equivalence.

discrepancy is that this transporter is expressed on normal brain capillary endothelial cells, but its expression was impaired during the establishment of immortalized MBEC4.

The characteristics of choline transport, such as kinetic parameters, functional properties, and effects of inhibitors, were well preserved between *in vivo* and MBEC4 *in vitro,* and were similar to those of the OCT family. Therefore, MBEC4 may be a useful *in vitro* model to investigate the function and molecular characteristics of OCT on the BBB.

In conclusion, we have confirmed the existence of a choline transporter, which is dependent upon membrane potential, but independent of Na^+ and pH, on brain capillary endothelial cells in mice by using an *in situ* brain perfusion technique. This choline transporter may contribute to the distribution of various basic drugs and neurotransmitters into the brain. The similarities in functional characteristics to those of renal proximal tubules strongly support the idea that the choline transporter at the BBB is one or more of the isoforms of the organic cation transporter (OCT) family. This choline transporter at the BBB *in vivo* showed characteristics almost identical to those of the transporter in MBEC4.

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