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Research Article

Study on Brain Interstitial Fluid Distribution and Blood-Brain Barrier Transport of Baclofen in Rats by Microdialysis

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Purpose. This study was performed to examine the distribution in the brain interstitial fluid (ISF) and the blood-brain barrier (BBB) transport of baclofen in rats by a microdialysis technique.

Methods. Following an *i.v.* bolus administration and/or the constant *i.v.* infusion of baclofen to the microdialysis cannula-bearing anesthetized rats, the concentrations of baclofen in the hippocampal ISF, whole brain tissue, cerebrospinal fluid (CSF), and plasma were determined by high-performance liquid chromatography (HPLC). Data were kinetically analyzed to estimate the transport parameters, *i.e.*, the influx clearance (CL_{in}) from plasma to brain and the efflux rate constant (k_{eff}) from brain to plasma, and the steady-state volume of distribution in the brain (V_d).

Results. The concentrations of baclofen in ISF, whole brain tissue, and CSF at the pseudo-steady state were almost 30-fold lower than the plasma unbound concentration, suggesting the restricted distribution of baclofen in the brain. The estimated values of CL_{in} and k_{eff} were 0.00157 ± 0.00076 ml/min/g of brain and 0.0872 ± 0.0252 min^{-1}, respectively. The efflux clearance (CL_{out}) calculated by multiplying k_{eff} by V_d (0.816 ± 0.559 ml/g of brain) was 0.0712 ± 0.0529 ml/min/g of brain, and it was significantly 40-fold greater than the CL_{in} value and fully greater than the convective flow in ISF. Furthermore, no significant concentration gradient was observed between ISF and CSF. These results suggest that the CL_{out} value mainly reflects the efflux clearance through the BBB. Additionally, the hippocampal ISF/plasma concentration ratio of baclofen was markedly increased by both systemic administration of probenecid and its direct instillation into ISF.

Conclusions. The restricted distribution of baclofen in the brain ISF may be ascribed to the efficient efflux from the brain through the BBB which is regulated possibly by a probenecid-sensitive organic anion transport system.

KEY WORDS: baclofen; blood-brain barrier; microdialysis; organic anion transport system; probenecid.

INTRODUCTION

Baclofen [4-amino-3-(4-chlorophenyl)butyric acid] is a GABA derivative that is used clinically as a centrally acting antispastic agent (1). Furthermore, baclofen has been shown to be a specific $GABA_B$ receptor agonist, which has inhibitory effects on the release of the classical neurotransmitters: noradrenaline, dopamine, and serotonin (2). Recently, baclofen has been found to inhibit the release of endogenous excitatory (glutamine, aspartate) and nonexcitatory (taurine, alanine) amino acids by high KCl stimulation from rat frontal cortex slices *in vitro* (3). This drug is also a potential candidate as a test substrate to examine the brain function. However, the distribution of baclofen in brain ISF, which is important for the binding to $GABA_B$ receptor, has not been clarified. Generally, the drug concentration in the brain ISF has been known to be regulated predominantly by the influx and efflux transport processes across the BBB, an intracellular-to-ISF partition, and the diffusional transfer process between ISF and cerebral ventricles (4,5). In order to elucidate and predict the distribution in brain ISF, therefore, it is necessary to evaluate quantitatively the above processes.

Recent reports on the BBB transport of baclofen have shown that, using a monolayer of bovine brain endothelial cells, baclofen is transported from the luminal side into the brain by a carrier-mediated transport system (6). Moreover, the presence of stereoselective transport of baclofen through the BBB has been reported (7). However, there have been no reports on the efflux transport process out of the brain through the BBB.

The currently developed microdialysis technique has been extensively used to directly determine endogenous and exogenous compounds in tissue ISFs in the fields of neurochemistry, physiology, and pharmacology (8,9). Moreover, this technique would be more advantageous to investigate the *in vivo* BBB transport of drugs, because the microdialysis probe is implanted in the ISF around the brain microvessel (10–13). Therefore, the measurement of baclofen in brain

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ISF would provide crucial information not only on its brain ISF distribution but also on the *in vivo* microvascular exchange processes across the BBB.

In this study, therefore, we examined the distribution in the brain ISF and the BBB transport of baclofen in rats by the microdialysis technique.

METHODS

Materials

Racemic baclofen was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

Animals

Adult male Wistar rats weighing 250–300 g were purchased from Nihon SLC (Shizuoka, Japan); they were housed, three or four per cage, in a laboratory with free access to food and water, and maintained on a 12-hr dark/12-hr light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%).

Microdialysis Procedure

Surgical procedures for implantation of the transcranial-typed microdialysis cannula have been described by Terasaki *et al.* (11). The microdialysis cannula was constructed from Cuprophan hollow-fiber (i.d., 0.2 mm; wall thickness, 11 μm; MW cut-off, 12500; RENAK-E, RE-10M, Kawasumi Chemical Industries Ltd., Tokyo, Japan) under a zoom stereomicroscope (EMZ-TR, Meiji Techno Co. Ltd., Tokyo, Japan), and stainless steel tubing (o.d., 0.2 mm; MT Giken, Tokyo, Japan), as described by Deguchi *et al.* (14). The distance between the stainless steel tubings, *i.e.* the length of the diffusible part of the probe, was 8 mm.

The microdialysis cannula was implanted in the hippocampus of ketamine (235 mg/kg, i.m.)-anesthetized rats. The rat's head was held in a stereotaxic apparatus (SR-6, Narishige Scientific Instrument Lab., Tokyo, Japan), and 1.0-mm holes were made on both sides of the skull, 3.4 mm posterior to the bregma and 3.5 mm below the dura, using a dental drill (MINITOR, Narishige Scientific Instrument Lab.). The microdialysis cannula was passed through the holes while Krebs-Ringer phosphate buffer (KRP buffer; 120 mM NaCl, 2.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 1.4 mM $Na_2HPO_4 \cdot 12H_2O$, 0.3 mM L(+)ascorbic acid, pH 7.4) was perfused into the cannula at a constant flow rate of 5 μl/min, and then the cannula was secured in place with dental cement (Fuji I, GC Corporation, Tokyo, Japan). The cannula-bearing rat was allowed free access to food and water for 48 h, and then they were used in the microdialysis experiments.

Study Design and Drug Administration

To perform the brain microdialysis, the cannula-bearing rat was anesthetized with ketamine (118 mg/kg). Subsequently, the left femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho Ltd., Tokyo, Japan) filled with heparin-saline solution (100 units/ml), and the animals were tracheotomized with polyethylene tubing (SP-120), for mechanical ventilation. The body temperature was kept constant at 37°C by means of a heating lamp throughout the experiments.

One side of the microdialysis cannula was connected with a polyethylene tubing (SP-10) and perfused with KRP buffer at a constant flow rate of 5.0 μl/min using an infusion pump (Model 22, Harvard Apparatus, South Natick, MS). At 30 min after initiation of perfusion, a bolus dose (50 mg/kg) of baclofen was administered intravenously *via* a femoral vein. Subsequently the animals were artificially ventilated using a rodent respirator (Model 681, Harvard Apparatus, South Natick, MS). The dialysate samples were collected at 20-min intervals for 3 hr. Blood was withdrawn through the catheter at 5, 15, 30, 60, 120, and 180 min.

For the determination of baclofen in brain tissues and CSFs, rats were anesthetized with ketamine (235 mg/kg), and a bolus dose (50 mg/kg) of baclofen was administered as described above. At 1, 2, 3, 4, 15, 30, 60, 120, and 180 min after the drug administration, blood was withdrawn through the catheter *via* a femoral artery. Then rats were decapitated, and the brain tissue was obtained after the removal of choroid plexus. Immediately, tissues were rinsed with cold saline, blotted, and weighed. CSF samples (100 μl) were taken by cisternal puncture technique (16) at 15, 30, 60, 120, and 180 min.

Additionally, intravenous infusion studies were carried out, to obtain the steady-state concentration of baclofen in the rat brain, as follows. A bolus dose of baclofen (20.9 mg/kg) was administered intravenously *via* a catheter, followed by immediate infusion at a constant rate of 141.4 μg/min/kg. At 180 min after the initiation of infusion, blood and brain tissues were obtained as described above.

Effect of Probenecid on Plasma-Brain Transport of Baclofen

To examine the effect of probenecid on plasma-brain transport of baclofen, the following two experiments were conducted.

In the first experiment, a 20 mg/kg *i.v.* bolus injection and a 35 mg/kg/hr *i.v.* infusion of probenecid were initiated *via* a femoral vein cannula, at 60 min before 50 mg/kg *i.v.* administration of baclofen. The dialysate samples were collected at 20-min intervals for 3 hr. Blood samples were withdrawn through the catheter at 5, 15, 30, 60, 120, and 180 min.

In the second experiment, the dialysis probes were initially perfused with KRP buffer for 60 min after a 50 mg/kg *i.v.* administration of baclofen. Thereafter, the dialysis solutions were switched from KRP buffer solutions to isotonic solutions containing 30 mM probenecid (30 mM probenecid, 144 mM Na^+, 2.4 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 121.2 mM Cl^-, 300 mOsm, pH 7.4) or 120 mM probenecid (144 mM Na^+, 2.4 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 31.2 mM Cl^-, 300 mOsm, pH 7.4), and they were perfused through the dialysis probe for the subsequent 120 min. Blood and dialysate samples were collected as described above.

Determination of *in Vitro* Permeability Rate Constants of Baclofen, Probenecid, and Antipyrine

The KRP buffer containing baclofen (100 μM), probenecid (350 μM), or antipyrine (100 μg/ml) was used as the reservoir solution. The dialysis cannula was placed into 30

ml of the reservoir solution kept at 37°C, and the dialysis was performed with the KRP buffer solution at a constant flow rate of 5 μl/min. The dialysate was collected at 20-min intervals for 80 min, and the concentrations of baclofen, probenecid, and antipyrine in the dialysate (C_d) and the reservoir solution (C_r) were determined by the HPLC method described below. The *in vitro* permeability rate constant (PA_{vitro}) was estimated from the following relationship (14).

$$\frac{C_d}{C_r} = F\{1 - \exp(-PA_{vitro}/F)\} \quad (1)$$

where F is the dialysis flow rate.

Determination of the Unbound Fraction in Plasma

The plasma unbound fraction of baclofen was determined by the same ultrafiltration technique as described by Deguchi *et al.* (16).

Analytical Procedure

The concentrations of baclofen in dialysate, plasma, CSF, and brain tissue were determined by a slight modification of the HPLC method of Bree *et al.* (7).

For deproteinization, 30 μl of a plasma sample was mixed with 60 μl of methanol; this was allowed to stand for 60 min at −20°C; then it was centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was diluted with an appropriate volume of distilled water. A portion of the sample was allowed to react with an equal volume of OPA reagent (50 mg of *o*-phthalaldehyde, 0.9 ml of methanol, 0.1 ml of 0.4 M borate buffer (pH 9.2), and 50 μl of 2-mercaptoethanol) for 90 sec. A 30-μl aliquot was injected onto an HPLC column.

Brain tissues were homogenized with a 4-fold volume of 50% MeOH in a glass homogenizer in an ice-cold bath, and they were allowed to stand for 8 hr at −20°C. The reaction procedure was the same as for the plasma sample.

Dialysate and CSF samples were directly allowed to react with OPA reagent without deproteinization after appropriate dilution, and a 30-μl aliquot was injected onto an HPLC column.

The HPLC system consisted of a pump (880-PU, Japan Spectroscopic Co. (Jasco), Tokyo, Japan), a fluorescence detector (FP210, Jasco), and an integrator (Chromatocorder 12, System Instruments, Co. Ltd., Tokyo, Japan). The HPLC analytical column was a Finepak SIL $C_{18}S$ ODS (4.6 mm I.D. × 25 cm length, 5-μm particle size, Jasco), and the guard column was a μ-Bondapak C_{18}, Guard-Pak Insert (Waters, MA). The flow rate was 1.0 ml/min, and the column eluate was monitored fluorometrically at excitation and emission wavelengths of 368 and 434 nm, respectively. The mobile phase was 0.1 M acetate buffer (pH 6.95): methanol : tetrahydrofuran = 525 : 455 : 20 (v/v/v) for dialysate, CSF, and plasma analysis, and 550 : 430 : 20 (v/v/v) for brain tissue analysis. The peak area was used for quantification. The concentration was determined from the calibration curve prepared by the same procedure as that for the respective sample. The linear relationship was obtained within the range of 0.1 μM to 2.0 μM, and the detection limit was 0.1 μM. The brain volume was calculated from the weight of the brain sample as assumed specific gravity of 1.0 g/ml of brain.

Probenecid in dialysate and plasma samples was assayed according to the method reported by Galinsky *et al.* (17). The HPLC system was the same as described above. The detection limit was 0.35 μM.

Data Calculation

Estimation of Concentration in Brain ISF

The unbound concentrations of baclofen and probenecid in brain ISF ($C_{isf,u}$) were estimated according to the reference method using antipyrine (11,14,16).

$$C_{isf,u} = C_d/\{1 - \exp(-R_{d,ref}PA_{vitro}/F)\} \quad (2)$$

where C_d is the concentration in dialysate determined by brain microdialysis. $R_{d,ref}$ is the effective dialysis coefficient of the reference compound, antipyrine, which is the ratio of the *in vivo* and *in vitro* permeability rate constants of antipyrine, PA_{vivo}, and PA_{vitro}, respectively. In this study, the PA_{vivo} value was determined using the following relationship, from the plasma concentrations and the dialysate concentrations collected by brain microdialysis after a 100-mg/kg *i.v.* administration of antipyrine.

$$\frac{Cd_{t1}^{t2}}{\overline{Cp}} = F\{1 - \exp(-PA_{vivo}/F)\} \quad (3)$$

where Cd_{t1}^{t2} is the concentration in dialysate collected from time t1 to time t2, and $\overline{Cp}$ is the plasma concentration collected at the midpoint from time t1 to time t2. The concentrations of antipyrine in dialysate and plasma were determined by an HPLC method described by Deguchi *et al.* (14). Total concentration in ISF (C_{isf}) was supposed to be equal to $C_{isf,u}$, because of a very low protein content in brain ISF (9).

Estimation of Transport and Distribution Parameters

Plasma-brain transport parameters, *i.e.*, the influx clearance (CL_{in}) from plasma to brain and the efflux rate constant (k_{eff}) from brain to plasma, were estimated by fitting the brain concentration-time profile to an integrated form of the two-compartment single membrane model.

$$\frac{dX_{br}}{dt} = CL_{in}f_pC_{p,t} - k_{eff}X_{br} \quad (4)$$

where X_{br} is the amount per g of brain as corrected by the amount remaining in the vascular space. f_p is the unbound fraction in plasma. $C_{p,t}$ is the calculated plasma concentration at time t, which was obtained by fitting the observed plasma concentrations to the two-compartment model by nonlinear regression analysis.

$$C_{p,t} = 360.7\, e^{-0.0694t} + 211.7\, e^{-0.00428t} \quad (5)$$

This expontential equation for $C_{p,t}$ was used as an input function in eq. (4).

For the fitting procedure, the initial value of CL_{in} was taken from the slope of the integration plot analysis (18) based on the data of initial uptake by brain.

$$\frac{Am}{C_{p,t}} = CL_{in}\frac{\int_0^t f_p C_p(\tau)d\tau}{C_{p,t}} + V_i \quad (6)$$

where Am is the total amount of baclofen per g of brain, V_i is the functional volume of distribution that rapidly exchanges with plasma. This value was used to estimate C_{br} by subtracting the amount remaining in the vascular space ($V_i \times C_{p,t}$) from Am. The initial value of k_{eff} was estimated from the amount in brain and the concentration in plasma at steady state ($X_{br,ss}$ and $C_{p,ss}$, respectively) as follows.

$$k_{eff} = \frac{CL_{in} f_p C_{p,ss}}{X_{br,ss}} \quad (7)$$

The fitting was performed by a nonlinear least-squares program with the Bayesian algorithm (MULTI2(BAYES)) (30) without weighting. The initial value of CL_{in} was constrained within the calculated SD of initial value, since the integration plot analysis has been demonstrated to provide a reliable estimate of CL_{in} value.

To estimate the efflux clearance (CL_{out}) from brain to plasma, the steady-state volume of distribution in brain (V_d) was defined as follows.

$$V_d = \frac{X_{br,ss}}{C_{isf,ss}} \quad (8)$$

where $C_{isf,ss}$ is the concentration in ISF at steady state. Then, CL_{out} was calculated by multiplying k_{eff} by V_d.

$$CL_{out} = k_{eff} V_d \quad (9)$$

Statistical Analysis

All data are presented as mean ± SE except as otherwise noted. Student's *t* test was used to compare individual means.

RESULTS

In Vitro Permeability Rate Constant (PA_{vitro})

PA_{vitro} values of baclofen, antipyrine, and probenecid were determined from Eqn. 1. *In vitro* probe recoveries of baclofen, antipyrine, and probenecid were 13.1 ± 0.89% (n = 6), 14.3 ± 0.3% (n = 6), and 11.9 ± 1.1% (n = 3), respectively, and their PA_{vitro} values were estimated to be 0.702 ± 0.051 μl/min (n = 6), 0.733 ± 0.028 μl/min (n = 6), and 0.633 ± 0.063 μl/min (n = 3), respectively.

Dialysis Coefficient (R_d) of Antipyrine

To extrapolate the concentration of baclofen in ISF by the reference method, the R_d value of antipyrine was determined by Eqn. 3, from concentrations in the dialysate and plasma after a 100-mg/kg *i.v.* bolus injection of antipyrine. The value was 0.421 ± 0.011 (n = 42), which is almost identical to the steady-state value (0.389 ± 0.011) reported by Terasaki *et al.* (11).

Plasma and ISF Concentrations of Baclofen

Figure 1 (open and closed circles) shows concentration-time profiles in the plasma and ISF following a 50-mg/kg *i.v.* administration of baclofen. Plasma concentrations declined biexponentially. No appreciable plasma protein binding was observed (fp = 1.01 ± 0.04 (n = 6)). The average concentration of baclofen in ISF, extrapolated from the dialysate concentration in the initial 20-min fraction,was 19.5 ± 4.4 μM, which was 30-fold lower than the plasma concentration. Thereafter, the concentration in ISF declined in parallel with that in plasma. The average concentration in ISF at the pseudo-steady state (100–140 min after the administration) was 6.06 ± 1.0 μM and it was about 27-fold lower than the plasma concentration.

Brain Tissues and CSF Concentrations

Figures 2 (A) and (B) show concentration-time profiles in plasma, whole brain tissues, and CSF following a 50 mg/kg *i.v.* administration of baclofen, respectively. The pseudo steady-state concentrations in brain tissue and CSF at 120 min after the administration of baclofen were 5.70 ± 0.45 μM and 4.85 ± 0.60 μM, respectively, and these values were 30-fold lower than the plasma concentration (175 ± 37 μM). No significant concentration gradients were observed between brain tissue and ISF, or CSF and ISF. On the other hand, the steady-state concentrations of baclofen in plasma and brain obtained from the infusion study were 250.0 ± 31.4 μM (n = 3) and 9.33 ± 2.19 (n = 3) μM, respectively.

Estimation of CL_{in}, CL_{out}, k_{eff} and V_d

Figure 3 shows the result of the integration plot analysis by Eqn. 6 on the basis of the data of initial uptake by brain following a 50 mg/kg *i.v.* administration of baclofen. The slope of the line (CL_{in}) with the linear regression was 0.00169 ± 0.00052 ml/min/g of brain (mean ± calculated SD) and the ordinate intercept (V_i), was 0.0102 ± 0.00149 ml/g of brain (mean ± calculated SD). On the other hand, the value of k_{eff} calculated by Eqn. 7 from the amount in brain and the concentration in plasma at steady state was 0.0809 ± 0.0561 ml/min/g of brain (mean ± SD, n = 3). Using these values as initial estimates, the brain concentrations shown in Fig. 2(C)

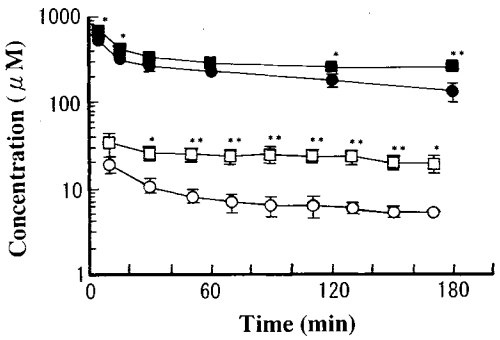

Fig. 1. The concentrations of baclofen in plasma (closed symbols) and hippocampal ISF (open symbols) versus time curves after a 50 mg/kg *i.v.* bolus administration to rats. Baclofen was injected into the femoral vein in control (●, plasma; ○, ISF) and probenecid (20 mg/kg *i.v.* bolus and 35 mg/hr/kg *i.v.* infusion doses)-pretreated (■, plasma; □, ISF) rats. Each point represents the mean ± S.E. of four to nine rats. * $p < 0.05$ and ** $p < 0.01$ vs control in Student's t-test.

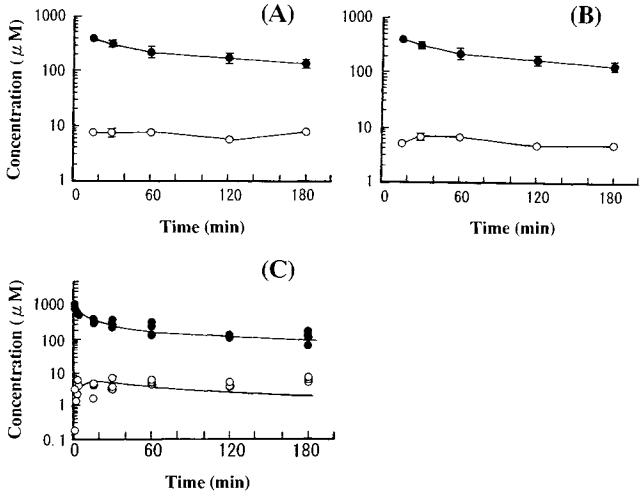

Fig. 2. (A) The concentrations of baclofen in plasma (●) and whole brain tissue (○) versus time curves after a 50-mg/kg *i.v.* bolus administration to rats. Each point represents the mean ± S.E. of three to four rats. (B) The concentrations of baclofen in plasma (●) and CSF (○) versus time curves after a 50-mg/kg *i.v.* bolus administration to rats. Each point represents the mean ± S.E. of three to four rats. (C) Time profiles for plasma (●) and brain (○) concentrations of baclofen after a 50-mg/kg *i.v.* bolus administration to rats. Brain concentration was corrected by the amount remaining in the intravascular space. The data were taken from Fig. 2(A) and Fig. 3, and are plotted individually. Solid line shows the computer-generated simulation curve using CL_{in} (0.00157 ml/min/g of brain) and k_{eff} (0.0872 min^{-1}) that were estimated by fitting the pooled data of 24 rats to Eqn. 4.

was fitted to Eqn. 4 by a nonlinear least-squares regression analysis. The final estimates for CL_{in} and k_{eff} are given in Table I.

Furthermore, the V_d and CL_{out} values were calculated from Eqns. 8 and 9, respectively, and they are given in Table I. CL_{out} was significantly 40-fold greater than CL_{in} ($p < 0.001$).

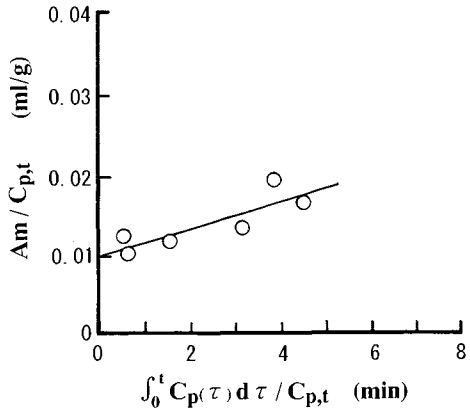

Fig. 3. An integration plot of the initial uptake of baclofen by brain over 1 to 4 min after a 50 mg/kg *i.v.* bolus administration to rats. The slope of the line (CL_{in}) and the ordinate intercept (Vi) were estimated to be 0.00169 ± 0.00052 ml/min/g of brain (mean ± calculated SD) and 0.0102 ± 0.00149 ml/g of brain (mean ± calculated SD), respectively.

Table I. Parameters for the Plasma-Brain Transport and the Distribution in Brain of Baclofen in Rats

Parameters	
CL_{in} (ml/min/g of brain)	0.00157 ± 0.00076[a]
CL_{out} (ml/min/g of brain)	0.0712 ± 0.0529[bd]
k_{eff} (min^{-1})	0.0872 ± 0.0252[a]
V_d (ml/g of brain)	0.816 ± 0.559[c]

[a] The value represents the mean ± calculated SD estimated by fitting the pooled data of 24 rats to eq. (4).
[b] The value was calculated by multiplying k_{eff} by V_d. The SD of this estimate was calculated by the principle of error deviation.
[c] This value was calculated from Eq. (8). The value represents the mean ± SD of 3 rats.
[d] Significantly different from CL_{in} ($p < 0.001$).

Effects of Probenecid on ISF Concentrations of Baclofen

Concentrations of baclofen in plasma and hippocampal ISF following the systemic coadministration of probenecid are shown in Fig. 1 (open and closed squares). The plasma concentrations of baclofen during the probenecid infusion were slightly higher than those in control rats. On the other hand, the ISF concentrations at the pseudo-steady state were 3 times higher than those observed in control rats. The average concentrations of probenecid in plasma and ISF during 0–180 min after the bolus administration of baclofen were 554 ± 59 μM (mean ± S.E., n = 8) and 48.0 ± 6.4 μM (mean ± S.E., n = 18), respectively.

Fig. 4 shows the results of experiments in which 30 mM or 120 mM probenecid was perfused through the microdialysis probe. The plasma levels of baclofen were almost equal to those in control rats. However, the concentrations of baclofen in ISF increased gradually with time after switching the dialysis solutions from KRP to isotonic solutions containing

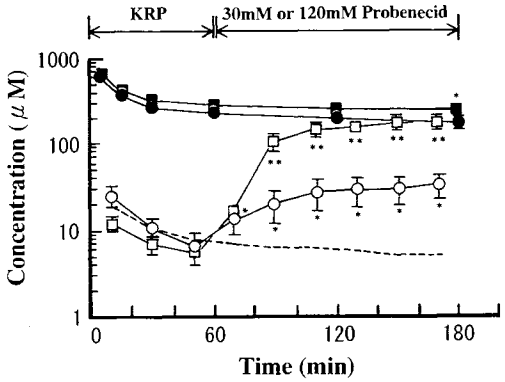

Fig. 4. Effects of probenecid on the concentrations of baclofen in plasma and hippocampal ISF in rats. The dialysis probes were initially perfused with KRP (pH 7.4) for 60 min after a 50 mg/kg *i.v.* administration of baclofen. Thereafter, the dialysis solutions were switched from KRP to isotonic solution containing 30 mM or 120 mM probenecid, and they were perfused through the dialysis probe for the subsequent 120 min. Each time period is indicated at the top of the panel. The dotted line represents the concentration level in hippocampal ISF in control rats in Fig. 1. Each point represents the mean ± S.E. of three rats. Key: ((●) plasma and (○) ISF, 30 mM probenecid); ((■) plasma and (□) ISF, 120 mM probenecid). * $p < 0.05$ and ** $p < 0.001$ vs control in Student's t-test.

30 mM or 120 mM probenecid, and they reached 33 μM (by 30 mM probenecid) and 170 μM (by 120 mM probenecid) at 120 min after the initiation of perfusion, respectively, depending on the probenecid concentration. In these experiments, the maximum concentrations of probenecid in brain ISF, estimated from in the *in vivo* permeability rate constant (R_d PA_{vitro}) of probenecid, were 1.7 mM and 6.9 mM, respectively.

DISCUSSION

This study was designed to examine the brain ISF distribution and the BBB transport of baclofen in rats using the brain microdialysis technique. The major findings obtained in the present study were: (a) the concentration of baclofen in brain ISF at the pseudo-steady state was 27-fold lower than that in plasma; (b) the brain-to-plasma efflux clearance is larger than the plasma-to-brain influx clearance; (c) the concentration in brain ISF increased significantly with probenecid administration, a potent inhibitor of the active organic anion transport system.

In the present study, we herein used a "reference method" to estimate the concentration in ISF from that in dialysate. This method has been demonstrated to provide a reliable estimate of the steady-state unbound concentration in ISF of muscle, liver, lung, and brain (11,14,16). Since Eqn. 2 was derived under the steady-state conditions, it is important to examine whether this method is applicable to the determination of baclofen in ISF after its bolus *i.v.* injection. The effective dialysis coefficient (R_d value) of antipyrine after a 100-mg/kg bolus *i.v.* injection, which was used to estimate the PA_{vivo} value from the PA_{vitro} value of baclofen, was 0.421 ± 0.011 (mean ± S.E.; ranging from 0.334 to 0.630). The value was in good agreement with that obtained at steady-state conditions (0.389 ± 0.011; mean ± S.E.) (11). In addition, Terasaki *et al.* and Deguchi *et al.* (11,14,16), have found that the R_d value is insensitive to both the molecular weight and the plasma membrane permeability of the substrates, whereas the ISF volume is the most sensitive factor determining the R_d value. These findings suggest that the "reference method" is satisfactorily applicable to various drugs. Fortunately, baclofen and antipyrine have similar molecular weights and volumes of distribution in the brain. Accordingly, the "reference method" using antipyrine would not produce significant errors between real and estimated concentrations of baclofen in brain ISF.

As a rule, it has been recognized that the unbound drug concentration in tissue ISF is important to characterize the *in vivo* binding to receptors and the subsequent pharmacological responses. As shown in Fig. 1, the concentration of baclofen in the rat hippocampal ISF reached a maximum level at 10 min after a bolus administration, and then it decreased in parallel with the plasma concentration. The concentration level in ISF at the pseudo-steady state was 27-fold lower than that in plasma. This suggests that the concentration of baclofen in the brain ISF, which is available for the binding to $GABA_B$ receptors, is low compared with that in other peripheral tissues. Therefore, clarification of the predominant factors responsible for this restricted distribution of baclofen in brain ISF would provide substantial insight into the binding to $GABA_B$ receptor and pharmacological responses, such as inhibition of the release of amino acids.

The concentration of a substance in brain ISF is regulated by plasma protein binding, the BBB transport rates between plasma and ISF, intracellular-to-ISF distribution, and metabolism in this space (4). Furthermore, the diffusion rate from the ISF space toward the CSF pool (5), and the convective flow of ISF (20), may in part contribute to the concentration level in ISF. Accordingly, we measured the transport clearances between plasma and brain and the volume of distribution in brain (see Table I). The obtained value of CL_{in} (0.00157 ml/min/g of brain) was smaller than the cerebral plasma flow (0.515 ml/min/g of brain (19)), suggesting that CL_{in} approximates the BBB permeability-surface area (PS) product. The CL_{out} value calculated by multiplying k_{eff} and V_d was 0.0712 ml/min/g of brain. This value was 40-fold greater than CL_{in} and fully greater than the convective flow in ISF (0.0001 ml/min/g of brain (20)). In addition, no significant concentration gradient was observed between ISF and CSF, indicating that the diffusional transfer from ISF to CSF would little contribute to the CL_{out} value. Furthermore, the observed V_d value of baclofen was comparable to the reported value of antipyrine (19), suggesting that baclofen is not localized in ISF space but distributes entirely into water space in the brain. These results suggest that the CL_{out} value mainly reflects the efflux clearance through the BBB and the BBB transport of baclofen may be asymmetric, and thus, the major cause of the restricted distribution of baclofen in the brain ISF appears to be this BBB transport property.

The finding that CL_{out} exceeds CL_{in} may suggest the existence of the active efflux system that participate in the BBB transport of baclofen. Baclofen is a GABA derivative and possesses a carboxyl residue in its chemical structure, and so baclofen may be actively pumped out of the brain *via* an organic anion efflux mechanism as seen in choroid plexus for β-lactam antibiotics (21–24). Therefore, to test this hypothesis, we examined whether probenecid inhibits the efflux of baclofen out of the brain ISF. The ISF/plasma concentration ratio of baclofen at the pseudo steady-state was increased to 3-fold of that in control rats by intravenous co-administration of probenecid. Additionally, when probenecid was directly infused into brain ISF through the microdialysis probe, the concentration of baclofen in the hippocampal ISF was gradually and markedly increased with time, depending on the probenecid concentrations. These results strongly suggest the possibility that baclofen is transported out of the brain ISF by a probenecid-sensitive organic anion transport system. The hypothesis of an efficient efflux pump at the BBB is supported by the reports for valproic acid (25–27) and zidovudine (12,13). However, these results could be explained by assuming the probenecid-sensitive facilitated transport system at the BBB. That is to say, the influx transport from plasma to brain at physiological condition is competitively inhibited by endogenous organic anions and/or saturated by the high concentration of baclofen itself in plasma since the Michaelis constant (Km) to the transport carrier of baclofen has been reported to be 58.5 μM (6). In contrast, the efflux transport from brain ISF to plasma is efficiently functioning because of low concentrations of endogenous compounds and/or baclofen in brain ISF. This results in the reduced CL_{in} compared with CL_{out}, and may provide us an interpretation

for a series of effects of probenecid on the BBB transport of baclofen.

Recent reports suggest that p-glycoprotein localizing at the luminal side of the brain capillary, functions as the efflux pump of highly lipophilic drugs (28,29). Although we cannot rule out this possibility, taking into account the high sensitivity of the BBB transport to probenecid and the chemical structure as mentioned above, it is difficult to think that baclofen is pumped out of the brain *via* this functional protein. Further detailed study would be necessary to resolve this issue.

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