In Vivo and In Vitro Evidence of Blood-Brain Barrier Transport of a Novel Cationic Arginine-Vasopressin Fragment 4-9 Analog

SHUICHI TANABE, YASUYUKI SHIMOHIGASHI, YASUHISA NAKAYAMA, TAKASHI MAKINO, TSUGUMI FUJITA, TAKERU NOSE, GOZOH TSUJJMOTO, TERUO YOKOKURA, MIKIHIKO NAITO, TAKASHI TSURUO, and TETSUYA TERASAKI

Yakult Central Institute for Microbiological Research, Kunitachi-shi, Tokyo, Japan (S.T., T.M., T.Y.); Laboratory of Structure-Function Biochemistry, Department of Molecular Chemistry, Graduate School of Science, Kyushu University, Fukuoka, Japan (Y.S., T.F., T.N.); Division of Molecular Cell Pharmacology, National Children’s Medical Research Center, Setagaya-ku, Tokyo, Japan (Y.N., G.T.); Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan (M.N., T. Tsuruo); and Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (T. Terasaki)

Accepted for publication March 29, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

The blood-brain barrier (BBB) transport and metabolism of a novel arginine-vasopressin fragment 4-9 [AVP4-9, isoelectric point; (pl) = 9.2] analog, that is, cationic AVP4-9 (C-AVP4-9, pl = 9.8), were examined in vivo and in vitro. At 45 min after an i.v. administration to mice, the cerebrum-to-plasma concentration ratios were 3.8 nM (high affinity) and 45.7 µM (low affinity). 125I-labeled C-AVP4-9 showed single-phase saturable acid-resistant binding, with a Kd value of 16.4 µM. The acid-resistant binding of 125I-labeled C-AVP4-9 was significantly dependent on temperature and medium osmolality. The acid-resistant binding of 125I-labeled C-AVP4-9 was inhibited by dancyclcadaverine, pheynylarsine oxide (endocytosis inhibitors), 2,4-dinitrophenol (a metabolic inhibitor), and AVP4-9, poly(L-lysine), and protamine (cationic substances), but not by poly(L-glutamic acid) (an anionic peptide) and the V1 and V2 vasopressin receptor antagonists. In addition, the conversion of C-AVP4-9 to AVP4-9 in the cerebral homogenate was confirmed by HPLC and mass spectrometry. The present results demonstrate that C-AVP4-9 is transported through the BBB more effectively than AVP4-9, via absorptive-mediated endocytosis, and that C-AVP4-9 is converted to the neuroactive parent peptide, AVP4-9, in the cerebrum.

Arginine-vasopressin (AVP) fragment 4-9 [AVP4-9, isoelectric point; (pl) = 9.2; Fig. 1] is a stable major metabolite of AVP in the central nervous system (CNS; Burbach et al., 1983, 1993). It is well documented that AVP4-9 exerts a more potent memory-facilitative effect than AVP (Gaffori and De Weid, 1986; De Weid et al., 1987).

Previous studies indicated that a novel cationic AVP4-9 analog (C-AVP4-9; pl = 9.8, Fig. 1) possesses more potent memory-facilitative activity than its parent peptide, AVP4-9 (Tanabe et al., 1997). C-AVP4-9 was designed to be converted to the neuroactive parent peptide, AVP4-9, by the postproline cleaving enzyme (PPCE), which was previously demonstrated to be abundant in the CNS and scarce in the plasma (Yoshimoto et al., 1979).

To act on the CNS, drugs and neuropeptides should be transported through the blood-brain barrier (BBB), which consists of brain capillary endothelial cells possessing tight intercellular junctions. With regard to BBB transport of peptides, previous studies have reported the receptor-mediated endocytosis (RME) of peptides, such as insulin (Duffy and Partridge, 1987), anti-insulin receptor antibody (Pardridge et al., 1995; Wu et al., 1998), transferrin (Fishman et al., 1987; Skarlatos et al., 1995; Wu et al., 1998), and transferrin receptor antibody (Pardridge et al., 1991; Shin et al., 1995; Walus et al., 1996), and the absorptive-mediated endocytosis (AME) of cationic peptides, such as E-2078 (Terasaki et al., 1989),...
ebiradite (Shimura et al., 1991), histone (Pardridge et al., 1989), and cationized albumin (Pardridge et al., 1990). In addition, these reports demonstrated that the AME-transported peptides show a far higher maximal internalization capacity into the brain capillary than the RME-transported peptides.

Accordingly, it is of great interest to investigate the BBB transport mechanism of C-AVP₄₋₉, because C-AVP₄₋₉ is a cationic peptide at physiological pH.

The purpose of the present study is to evaluate the BBB transport of C-AVP₄₋₉ based on pharmacokinetic analysis and clarify the BBB transport mechanism of C-AVP₄₋₉. We also demonstrated the conversion of C-AVP₄₋₉ to AVP₄₋₉ by PPCE in the CNS.

**Materials and Methods**

**Radiolabeling of Peptides.** AVP₄₋₉ ([pGlu⁴,Cyt⁶,Arg⁸]vasopressin fragment 4-9, Sigma Chemical Co., St. Louis, MO) was labeled with ³⁵S. As an intermediate of ³²S-labeled AVP₄₋₉, 3-nitro-2-pyridinesulfenyl-AVP₄₋₉ was synthesized by a previously demonstrated method (Shimohigashi et al., 1992). ³⁵S[Cysteine (Amer sham Pharma cie Biotech, Buckinghamshire, UK) was used immediately after purification to remove dithiothreitol by HPLC (see below). 3-nitro-2-pyridinesulfenyl-AVP₄₋₉ was rereacted with 74 MBq of ³⁵S[Cysteine in 500 μl of distilled water at room temperature for 24 h. C-AVP₄₋₉ (American Peptide Company, Sunnyvale, CA) was labeled with ¹²⁵I by the chloramine T method (Hunter and Greenwood, 1962; Tanabe et al., 1997). The reaction mixtures of ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ were purified by HPLC as described below. The ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ obtained had specific activities of 22.6 to 46.0 TBq/μmol (purity >99.0%), respectively.

**HPLC Conditions.** To quantify the unchanged ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ in vivo and in vitro uptake studies, HPLC was used. For the separation, the column J’sphere (YMC Inc., Wilmington, NC) was used. In the studies using unlabeled peptides, the HPLC elution was performed with the following linear gradient: solvent B in A (v/v): from 0 to 5% for 15 min, from 5 to 15% for 10 min, from 15 to 40% for 5 min, holding 40% for 5 min, and from 40% to 0% for 5 min with equilibration for 10 min. The eluate was monitored by UV absorbance at 210 nm.

**In Vivo Pharmacokinetic Studies.** ³⁵S-labeled AVP₄₋₉ (130 KBq), ¹²⁵I-labeled C-AVP₄₋₉ (1110 KBq), or 370 KBq of ¹²⁵I-labeled BSA (144 KBq/μg; NEN Life Sciences, Boston, MA) was administered i.v. into the tail vein of normal male ICR mice (6–8 weeks old; CLEA Japan, Tokyo). At 1, 5, 15, 30, and 45 min after administration, mice were sacrificed, and the plasma, cerebrum, liver, kidney, heart, and lung were obtained. The unchanged ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ in the plasma and tissues were quantified as follows. Ice-cold 2% phosphoric acid (3× volume for plasma, 4× volume for cerebrum, and 10× volume for other tissues) was added, and the mixtures were homogenized with an Ultraturrax (IKA Labortechnik, Staufen, Germany). The homogenates were then centrifuged (14,000g, 4°C, 30 min), and 500-μl aliquots of the supernatants were analyzed by HPLC. In the ¹²⁵I-labeled BSA studies, plasma and tissues were homogenized with a 5× volume of 30% ice-cold trichloroacetic acid (TCA). After centrifugation (14,000g, 4°C, 30 min), the radioactivity in each pellet was measured as unchanged ¹²⁵I-labeled BSA by a gamma counter. The plasma concentrations [Cₜ(t)] of unchanged labeled peptides were normalized as a percentage of the injected dose per milliliter. The Cₜ(t) data were fitted to the function (1) by a nonlinear least-squares regression analysis using a computer program, MULTI (Yamaoka et al., 1980):

\[
C_t(t) = A e^{-at} + B e^{-bt}
\]

where A and B are the intercepts, and a and β are the rate constants describing the fast and slow compartments of clearance from plasma, respectively. As an index of the distribution of unchanged labeled peptides to the tissues, the apparent tissue-to-plasma concentration ratio (Kₐᵖₚ) defined by the radioactivity of unchanged radiolabeled peptides per gram of tissue divided by that per milliliter of plasma, was calculated (Terasaki et al., 1984). The BBB permeation clearances (PS/Vₜₚ) of labeled peptides were calculated using the function (2):

\[
C_{t,p}(t)/C_{t,p}(t) = PS/V_{WP} \times AUC(0-t)/C_{t,p}(t) + V_p/V_{WP}
\]

where Vₚ, Vₜₚ, and Cₜ(t) are the rapidly equilibrated distribution volume of labeled peptides, the cerebral parenchyma, and the apparent tissue-to-plasma concentration ratio (Kₐᵖₚ) of labeled peptides, respectively. PS/Vₜₚ values of labeled peptides were obtained from the initial slopes of Cₜ(t)/Vₚ(t) [Kₚ₋ₚ versus the area under the curve [AUC(0−t)] Cₜ(t) as an integration plot (Smith et al., 1987; Kakee et al., 1996; Yang et al., 1997)].

**Capillary Depletion Study.** The left and right renal artery and vein of mice were ligated under ether anesthesia before use. ¹²⁵I-labeled BSA (370 KBq), ³⁵S-labeled AVP₄₋₉ (130 KBq), or ¹²⁵I-labeled C-AVP₄₋₉ (1110 KBq) was i.v. administered into the tail vein of mice. At 30 min after administration, plasma and cerebrum were obtained. The cerebra were separated into the parenchyma and the capillary fractions by a method reported previously (Yang et al., 1997). The unchanged ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ in the parenchyma fraction were extracted two times with a 10× volume of ice-cold 50% phosphoric acid and a 10× volume of ice-cold acetonitrile. After centrifugation (3500g, 4°C, 1 h), the supernatants were lyophilized and suspended in 1 ml of ice-cold methanol. After further centrifugation (3500g, 4°C, 1 h), the supernatants were again lyophilized and reconstituted with 600 μl of 0.1% trifluoroacetic acid, and 500-μl aliquots were analyzed by HPLC. The unchanged ³⁵S-labeled AVP₄₋₉ and ¹²⁵I-labeled C-AVP₄₋₉ in the capillary fraction were extracted with 600 μl of 5% phosphoric acid, and 500-μl aliquots were analyzed by HPLC. The unchanged ¹²⁵I-labeled BSA in the parenchyma and the capillary fractions were precipitated by ice-cold 30% TCA (5× volume for the parenchyma fraction and 500 μl for the capillary fraction), and the radioactivity in each pellet was measured with a gamma counter.
In Vitro Internalization Studies Using MBEC4. MBEC4 cells (Tatsuta et al., 1992) were used. Monolayers of MBEC4 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS in 5% CO₂/95% air at 37°C. The internalization of 35S-labeled AVP₄-₉ and 125I-labeled C-AVP₄-₉ into MBEC4 was examined by a method reported previously (Terasaki et al., 1992) with minor modifications. Briefly, monolayers of MBEC4 cells cultured in 24-well dishes were washed five times with 1 ml of the incubation buffer [122 mM NaCl, 3 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 0.4 mM K₂HPO₄, 1.4 mM CaCl₂, 10 mM d-glucose, 10 mM HEPES, 0.1% BSA, 1 tablet/50 ml Complete (a protease inhibitor cocktail; Boehringer Mannheim, Mannheim, Germany), pH 7.4, 300 mOsm] at 37°C. They were then preincubated in 200 µl of incubation buffer at 37°C for 30 min. In the uptake studies for the studies of the concentration dependencies, the uptakes were initiated by adding 200 µl of incubation buffer containing 35S-labeled AVP₄-₉ (18.5 KBq) or 125I-labeled C-AVP₄-₉ (18.5 KBq), and 92.5 KBq of [3H]inulin (13.5 GBq/mmol, NEN Life Sciences) to MBEC4. [3H]Inulin was used as the extracellular space marker. The effects of medium osmolality on the uptakes of 35S-labeled AVP₄-₉ and 125I-labeled C-AVP₄-₉ were examined with hypertonic buffer (incubation buffer with 1.2 M sucrose, 1600 mOsM) substituted for the incubation buffer. The effects of endocytosis inhibitors, metabolic inhibitor, and several peptides on the uptake of 125I-labeled C-AVP₄-₉ were examined in the presence of 0.5 mM danylcadaverine (Sigma), 0.1 mM phenylarsine oxide (Aldrich Chemical Co., Milwaukee, WI), 1 mM 2,4-dinitrophenol (Wako Pure Chemical Industries, Osaka, Japan), 0.5 mM V₃ vasopressor receptor antagonist ([[(a-mercaptopropane-β,β-cyclopentamethylene propionic acid)O-methyl-Tyr2,Arg₈]vasopressin, Peninsula Laboratories, Belmont, CA), 0.5 mM V₂ vasopressin receptor antagonist ([a-adamantaneacetyl]O-ethyl-o-Tyr2,Val₈]aminobutyryl [-Arg₉]-vasopressin; Sigma), 0.5 mM AVP₉, 0.3 mM polyl-lysine (Sigma), 0.3 mM protamine (Wako Pure Chemical Industries), or 0.3 mM poly-L-glutamic acid (Sigma). In the studies of the concentration dependencies, various concentrations of mixtures of 35S-labeled AVP₄-₉ and unlabeled AVP₄-₉ or 125I-labeled C-AVP₄-₉ and unlabeled C-AVP₄-₉ were added with [3H]inulin (92.5 KBq) to MBEC4 before preincubation. At designated times after incubation, the incubation supernatants (unbound fraction) were recovered, and the cells were washed seven times with 1 ml of ice-cold incubation buffer. An acid wash technique was then used to remove the labeled peptide binding to the cell surface and to evaluate the amounts of labeled peptides internalized into MBEC4. The cells were incubated with 1 ml of ice-cold acetate-barbital buffer (28 mM CH₃COONa, 120 mM NaCl, 20 mM barbital, pH 3.0, 320 mOsm) for 10 min. The buffer was then recovered (acid-soluble fraction), and the cells were subsequently washed three additional times with 1 ml of acetate-barbital buffer. The cells remaining were obtained as the acid-resistant fraction and solubilized with 50 µl of NaOH (0.5 N) and 200 µl of 2% Triton X-100. Aliquots of unbound (100 µl), acid-soluble (500 µl), and solubilized acid-resistant (200 µl) fractions were analyzed by HPLC. The protein assay of the acid-resistant fraction was carried out with BCA Protein Assay Reagent (Pierce, Rockford, IL). In accord with Terasaki et al. (1989, 1992), the data on the acid-resistant binding are expressed as the cell-to-medium concentration ratios (cell/medium ratios), corrected for an extracellular space using [3H]inulin, as follows:

\[
\text{Cell/medium ratio} = \left[ \frac{125}{35} \text{S-R} - 125 \text{I or } 35 \text{S-S}} \times \frac{3}{3-\text{H}} \times \frac{3}{\text{H-S}} / \text{mg MBEC4 protein}[\%]^{\text{125}} \text{I or } 3^{5} \text{S-M/µl of medium}} \]

where ³H, ³⁵S, and ¹²⁵I are the radioactivities of unchanged [³H]inulin, ³⁵S-labeled AVP₄-₉, and ¹²⁵I-labeled C-AVP₄-₉, respectively; and -R, -S, and -M are the acid-resistant, the acid-soluble, and the unbound fractions, respectively. The maximal internalization capacity (B₉max) and the half-saturation constant (Kₛ) were estimated by MULTI (Yamaoka et al., 1980). The cell/medium ratios were statistically analyzed using the Kruskal-Wallis test and Dunnett's test.

Enzymatic Conversion of C-AVP₄-₉ to AVP₄-₉ by Mouse Cerebral Homogenate. The mouse cerebra were homogenized with a 4× volume of metabolism assay buffer (the incubation buffer without BSA and Complete). The PPCE activity in the mouse cerebral homogenate was determined by a method reported previously (Yoshimoto et al., 1979). After preincubation at 37°C for 10 min, C-AVP₄-₉ was incubated with PPCE derived from Flavobacterium meningosepticum (authentic PPCE, 0.1 U/ml; Seikagaku Corp., Tokyo, Japan) or the mouse cerebral homogenate (20 mg cerebrum/ml, equivalent to 1.5 × 10⁻⁵ U/ml) in 250 µl of metabolic assay buffer in the presence or absence of the PPCE-specific inhibitor N-benzoxycarbonyl-prolyl-prolyl (ZPP, 1 mM; Yakult Central Institute for Microbiological Research, Tokyo, Japan) at 37°C for designated times. The metabolic reaction was stopped by adding 250 µl of 2% phosphoric acid. After centrifugation (14,000g, 4°C, 10 min), 400-µl aliquots of supernatants were analyzed by electrospray ionization-liquid chromatography-mass spectrometry (ESI-MS/MS) using a Finnigan TSQ 7000 triple quadruple mass spectrometer with ESI-MS/MS interface (Thermo Quest Corp., San Jose, CA). The operating conditions were as follows: spray voltage, 4.5 kV; sheath gas pressure, 70 psi (nitrogen); auxiliary gas flow, 5 arbitrary units; heated capillary temperature, 200°C. Mass spectra were acquired every 3 s with a scanning m/z range of 50 to 150. The other conditions were as described for HPLC conditions. The Michaelis constant (Kₘ) and maximum velocity (V₉max) were estimated by MULTI (Yamaoka et al., 1980). The intrinsic clearance of the conversion of C-AVP₄-₉ to AVP₄-₉ (Cl₉int) was defined as the V₉max Value divided by the Kₘ Value.

Results

In Vivo Pharmacokinetic Studies. The plasma concentration of i.v. administered ¹²⁵I-labeled AVP₄-₉ and ³⁵S-labeled AVP₄-₉ and ¹²⁵I-labeled C-AVP₄-₉ were characterized by a biexponential function and reached apparent steady states at 15 min after administration (Table 1, Fig. 2). The K₉app values of both ³⁵S-labeled AVP₄-₉ and ¹²⁵I-labeled C-AVP₄-₉ were the highest in the kidney among the tissues examined (Figs. 3 and 4). The K₉app values of ¹²⁵I-labeled C-AVP₄-₉ in the liver and the lung were decreased compared with those of ³⁵S-labeled AVP₄-₉ (Fig. 3), whereas the K₉app values of ¹²⁵I-labeled C-AVP₄-₉ in the cerebrum were increased compared with those of ³⁵S-labeled AVP₄-₉ (Fig. 4). At 45 min after the administration, K₉app values of ³⁵S-labeled AVP₄-₉ and ¹²⁵I-labeled C-AVP₄-₉ in the cerebrum were 0.103 ± 0.019 and 0.330 ± 0.030 ml/g cerebrum, respectively (Fig. 4). The K₉app values of ¹²⁵I-labeled BSA in the cerebrum were maintained.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>¹²⁵I-labeled BSA</th>
<th>³⁵S-labeled AVP₄-₉</th>
<th>¹²⁵I-labeled C-AVP₄-₉</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µM)</td>
<td>57.2 ± 1.0</td>
<td>5.2 ± 0.4</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>35.2 ± 0.6</td>
<td>1.03 ± 0.05</td>
<td>1.42 ± 0.28</td>
</tr>
<tr>
<td>T₉/₂ (min)</td>
<td>0.02 ± 0.002</td>
<td>0.67 ± 0.18</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>β (min)</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>T₀/β (min)</td>
<td>11.7 ± 4.3</td>
<td>12.3 ± 2.3</td>
<td>12.3 ± 2.3</td>
</tr>
</tbody>
</table>

³⁵S-labeled BSA (37 pmol/mouse), ³⁵S-labeled AVP₄-₉ (2.8 pmol/mouse), and ¹²⁵I-labeled C-AVP₄-₉ (25.0 pmol/mouse) were administered intravenously into the mice. The plasma concentration profile of ¹²⁵I-labeled BSA was analyzed by a monoexponential function, whereas ³⁵S-labeled AVP₄-₉ and ¹²⁵I-labeled C-AVP₄-₉ data were analyzed by a biexponential function. Each value represents the mean ± S.E. of three or four mice.
at 0.01 ml/g cerebrum (Fig. 4). By the integration plots, the BBB permeation clearances of 125I-labeled BSA, 35S-labeled AVP4-9, and 125I-labeled C-AVP4-9 were estimated to be 6.26 x 10^{-6} ± 1.26 x 10^{-5}, 1.47 ± 0.09 x 10^{-4}, and 3.10 ± 0.35 x 10^{-4} ml/min/g cerebrum, respectively (Fig. 5).

Capillary Depletion Study. At 30 min after the i.v. administration of 125I-labeled BSA, 35S-labeled AVP4-9, and 125I-labeled C-AVP4-9 to the mice whose renal vessels were ligated, the distributions of labeled peptides in the capillary and the parenchyma fractions of cerebrum were examined. The plasma concentrations of 125I-labeled BSA, 35S-labeled AVP4-9, and 125I-labeled C-AVP4-9 in mice whose renal vessels were ligated were 0.9, 10, and 3 times greater than those in the normal mice, respectively (Fig. 5).

Time Courses of Acid-Resistant Binding of 35S-Labeled AVP4-9 and 125I-Labeled C-AVP4-9 to MBEC4. Unchanged 35S-labeled AVP4-9 and 125I-labeled C-AVP4-9 in the acid-resistant fraction at 60 min, quantified by HPLC, were 6.48 ± 0.32 and 68.7 ± 5.6% of total recovered radioactivities, respectively. The cell/medium ratios of [3H]inulin coexisting with 35S-labeled AVP4-9 (Fig. 6A) or 125I-labeled C-AVP4-9 (Fig. 6B) were increased in a slightly time-dependent manner. The acid-resistant binding of 35S-labeled AVP4-9 increased with time and reached apparent equilibrium at 30 min (Fig. 6A). The acid-resistant binding of 125I-labeled C-AVP4-9 increased time-dependently (Fig. 6B). The acid-resistant binding of 35S-labeled AVP4-9 and 125I-labeled C-AVP4-9 in the parenchyma fraction was 1.04 ± 0.13 x 10^{-2} ml/g cerebrum. The K_{app} values of 125I-labeled BSA, 35S-labeled AVP4-9, and 125I-labeled C-AVP4-9 in the parenchyma fraction were 35, 68, and 22 times higher than those in the capillary fraction, respectively.

Concentration Dependencies of Acid-Resistant Binding of 35S-Labeled AVP4-9 and 125I-Labeled C-AVP4-9. In the studies of concentration dependencies, 35S-labeled AVP4-9 showed two-phase saturable acid-resistant binding (Fig. 7). The B_{max} values of 35S-labeled AVP4-9 for the high- and low-affinity sites of MBEC4 were estimated to be 0.72 ± 0.63 pmol/mg protein and 26.5 ± 6.0 pmol/mg protein, respectively. The K_{D} values of 35S-labeled AVP4-9 for the high- and low-affinity sites of MBEC4 were estimated to be 3.81 ± 3.46 nM and 45.7 ± 11.4 µM, respectively. In contrast, 125I-labeled C-AVP4-9 showed single-phase saturable acid-resistant binding (Fig. 7). The B_{max} and K_{D} values of 125I-labeled C-AVP4-9 for MBEC4 were estimated to be 14.7 ± 4.1 pmol/mg protein and 16.4 ± 5.0 µM, respectively.
in the legend to Fig. 3. Each point represents the mean ± S.E. of three or four mice.

Acid-Resistant Binding of 125I-labeled C-AVP4-9

BBB permeation clearances of 125I-labeled BSA (37 pmol/mouse), 35S-labeled AVP4-9, and 125I-labeled C-AVP4-9 (23.0 pmol/mouse) was administered i.v. into the normal mice. The $K_{p, app}$ values of 125I-labeled BSA (●), 35S-labeled AVP4-9 (△), and 125I-labeled C-AVP4-9 (□) shown in Fig. 4 were plotted against the indicated $AUC(0-t)/C_p(t)$ values to estimate the BBB permeation clearances of each labeled peptide (integration plot). Each point represents the mean ± S.E. of three or four mice.

Effect of Temperature, Osminality, Endocytosis Inhibitors, Metabolic Inhibitor, and Several Peptides on Acid-Resistant Binding of 125I-Labeled C-AVP4-9

As shown in Table 3, the incubation of 125I-labeled C-AVP4-9 with MBEC4 at 4°C for 60 min resulted in a decrease in the acid-resistant binding to 10.4 ± 1.2% of the control value. When the osmolarity of the incubation buffer was increased from 300 to 1600 mOsm, the acid-resistant binding of 125I-labeled C-AVP4-9 decreased significantly (Table 3). The acid-resistant binding of 125I-labeled C-AVP4-9 to MBEC4 was significantly diminished by dancyleadaverine, phenylarsine oxide, and 2,4-dinitrophenol (Table 3); markedly inhibited by AVP4-9, poly(L-lysine), and protamine (Table 4); and unaffected by poly(γ-glutamic acid), V1 vasopressin receptor antagonist, and V2 vasopressin receptor antagonist (Table 4).

Enzymatic Conversion of C-AVP4-9 to AVP4-9

C-AVP4-9 was converted by authentic PPCE and the mouse cerebral homogenate to the metabolites eluted at 12.0 and 13.3 min (Fig. 8A) or 13.3 min (Fig. 8C) in the HPLC. The retention times of authentic B chain (arginyl-histidinyl-proline; Fig. 1), AVP4-9, and C-AVP4-9 were 12.0, 13.3, and 23.0 min, respectively, in the HPLC conditions used here. The C-AVP4-9 metabolites produced by the authentic PPCE and the mouse cerebral homogenate eluted at 13.3 min in the HPLC (Fig. 8, A and C) showed the same $m/z$ as protonated authentic AVP4-9 ([M + H]$^+$ = 775) and doubly protonated AVP4-9 ([M + 2H]$^{2+}$ = 388) by ESI-LC-MS. Based on the agreements of retention time in the HPLC and the mass spectrometric behavior, C-AVP4-9 metabolites eluted at 13.3 min in Fig. 8, A and C, were identified as AVP4-9 (ZZP (1 mM) completely inhibited the conversion of C-AVP4-9 to AVP4-9 by the authentic PPCE (Fig. 8B). That concentration of ZZP also significantly ($p < .001$ by Wilcoxon’s test) inhibited 49.3 ± 0.3% (mean ± S.E., n = 4) of C-AVP4-9 conversion to AVP4-9 by the cerebral homogenate (Fig. 8D). The authentic PPCE and the mouse cerebral homogenate showed saturable conversion rates of C-AVP4-9 to AVP4-9 with the $C_{l,in}$ values 2.47 ± 0.24 (Fig. 8A) and 2.10 ± 0.14 ml/U/min (Fig. 8B), respectively.

Discussion

It has been demonstrated that cationic peptides are effectively transported through the BBB and reach the parenchyma of the CNS via AME (Pardridge et al., 1991; Terasaki et al., 1992). To enhance the BBB permeability of AVP4-9 (PI = 9.2, Fig. 1), we combined the peptide composed of cationic amino acids, that is, a B-chain (Fig. 1), with the free cysteine residue of AVP4-9 and obtained C-AVP4-9 (PI = 9.8, Fig. 1). C-AVP4-9 was also designed to be converted to AVP4-9 in the CNS by PPCE, which is abundant in the CNS (Yoshimoto et al., 1979). It was previously demonstrated that C-AVP4-9 facilitates the memory of rodents more effectively than AVP4-9 (Tanabe et al., 1997). In the present study, the BBB transport of C-AVP4-9 and its conversion to AVP4-9 in the CNS were evaluated.

The $K_{p, app}$ values of 125I-labeled C-AVP4-9 in the liver and the lung were lower compared with those of 35S-labeled AVP4-9 (Fig. 3), whereas the $K_{p, app}$ values of 125I-labeled C-AVP4-9 in the cerebrum were clearly higher compared with those of 35S-labeled AVP4-9 (Fig. 4). These results suggest that the cationization of AVP4-9 decreases the peripheral distribution of modified molecule and increases the distribution of the molecule to the whole cerebrum, including the parenchyma, capillary, and plasma. To evaluate the BBB transport of neuroactive peptides, however, it is necessary to evaluate the distribution of peptides to the parenchyma of the cerebrum. Hence, we separated the cerebrum of the mice administered with the labeled peptides into the capillary and the parenchyma fractions by the capillary depletion method (Yang et al., 1997) and evaluated the distributions of labeled peptides to both fractions. We have demonstrated previously that the alkaline phosphatase activity and $K_{p, app}$ value of i.v. administered [125I]-labeled low-density lipoprotein (markers for the capillary) in the parenchyma fraction, which express contamination of the capillary in the parenchyma fraction, were about 10% of those in the whole cerebrum (Yang et al.,
presence of [3H]inulin (34.3 ± 10^-6 C-AVP4-9 (8.0 pmol/mouse) was administered i.v. into mice whose renal vessels were ligated. The cerebra were obtained at 30 min after the injection and separated into parenchyma and capillary fractions by the capillary depletion technique. Each value represents the mean ± S.E. of three or four mice.

**TABLE 2**

Apparent cerebrum-to-plasma concentration ratios of 125I-labeled BSA, [35S]AVP4-9, and 125I-labeled C-AVP4-9

<table>
<thead>
<tr>
<th>Condition</th>
<th>125I-labeled BSA (ml/g fraction)</th>
<th>35S-labeled AVP4-9</th>
<th>125I-labeled C-AVP4-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenchyma</td>
<td>1.04 × 10^-2 ± 0.13 × 10^-2</td>
<td>1.14 × 10^-1 ± 0.16 × 10^-1</td>
<td>4.06 × 10^-1 ± 0.36 × 10^-1</td>
</tr>
<tr>
<td>Capillary</td>
<td>2.98 × 10^-4 ± 0.17 × 10^-4</td>
<td>1.67 × 10^-3 ± 0.61 × 10^-3</td>
<td>1.86 × 10^-2 ± 0.67 × 10^-2</td>
</tr>
</tbody>
</table>

**Table 3**

Effects of temperature, osmolarity, endocytosis inhibitors, and metabolic inhibitor on the acid-resistant binding of 125I-labeled C-AVP4-9 to MBEC4

<table>
<thead>
<tr>
<th>Condition</th>
<th>Acid-Resistant Binding % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 7.4</td>
</tr>
<tr>
<td>Low temperature (4°C)</td>
<td>104 ± 1.2°</td>
</tr>
<tr>
<td>Hypertonic (1600 mOsM)</td>
<td>53.4 ± 14.9°</td>
</tr>
<tr>
<td>Dendrocladaverine (0.5 mM)</td>
<td>74.3 ± 3.7°</td>
</tr>
<tr>
<td>2,4-Dinitrophenol (1 mM)</td>
<td>26.0 ± 3.9°</td>
</tr>
<tr>
<td>Control†</td>
<td>100 ± 14.6</td>
</tr>
<tr>
<td>Phenylarsine oxide (0.1 mM)</td>
<td>44.4 ± 8.0°</td>
</tr>
</tbody>
</table>

After preincubation for 30 min, 125I-labeled C-AVP4-9 and [3H]inulin were incubated under the indicated conditions at 4° or 37°C for 60 min. Each value represents the percentage of acid-resistant binding of 125I-labeled C-AVP4-9 as cell/medium ratios versus the control value (mean ± S.E. of three or four separate experiments).

* Significant inhibition (p < .05).

† Significant inhibition (p < .001).

**Table 4**

Effects of peptides on the acid-resistant binding of 125I-labeled C-AVP4-9 to MBEC4

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Acid-Resistant Binding % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5.5</td>
</tr>
<tr>
<td>AVP4-9 (0.5 mM)</td>
<td>36.3 ± 3.4°</td>
</tr>
<tr>
<td>V1 antagonist (0.5 mM)</td>
<td>111 ± 1.3</td>
</tr>
<tr>
<td>V2 antagonist (0.5 mM)</td>
<td>103 ± 10.6</td>
</tr>
<tr>
<td>Poly-l-lysine (0.3 mM)</td>
<td>66.1 ± 6.7°</td>
</tr>
<tr>
<td>Poly-l-glutamic acid (0.3 mM)</td>
<td>69.6 ± 5.0°</td>
</tr>
<tr>
<td>Phenylarsine oxide (0.1 mM)</td>
<td>87.3 ± 3.7°</td>
</tr>
</tbody>
</table>

After preincubation for 30 min, 125I-labeled C-AVP4-9 and [3H]inulin were incubated with or without the indicated peptides at 37°C for 60 min. Each value represents the percentage of acid-resistant binding of 125I-labeled C-AVP4-9 as cell/medium ratios versus the control value (mean ± S.E. of three or four separate experiments).

* Significant inhibition (p < .05).

† Significant inhibition (p < .001).

In the capillary depletion studies, we ligated the renal vessels of mice before the i.v. administration of 125I-labeled BSA, [35S]AVP4-9, or 125I-labeled C-AVP4-9 to eliminate the distribution of these labeled peptides to the kidney, the tissue where 35S-labeled AVP4-9 and 125I-labeled C-AVP4-9 showed the highest K_p,app value among the tissues examined (Figs. 3 and 4), and to elevate the plasma concentrations and the cerebral distributions of the labeled peptides. With the ligation treatments, the plasma concentrations of 35S-labeled AVP4-9 and 125I-labeled C-AVP4-9 at 30 min were 10 times and 3 times higher than those in the normal mice, respectively (Fig. 2), and this treatment enabled us to quantify the unchanged 35S-labeled AVP4-9 and 125I-labeled C-AVP4-9 in the parenchyma and capillary fractions of the cerebrum by HPLC. Because it is widely accepted that BSA remains in the plasma in the cerebrum (Triguero et al., 1990), the plasma volume of the cerebral parenchyma fraction was estimated to be 1.04 ± 0.13 × 10^-2 ml/g cerebrum (K_p,app of 125I-labeled BSA in the parenchyma fraction).
Fig. 8. Enzymatic conversion of C-AVP$_{4-9}$ to AVP$_{4-9}$. C-AVP$_{4-9}$ (150 μM) was incubated with the authentic PPCE (0.1 U/ml, A and B) or mouse cerebral homogenate (20 mg cerebrum/ml equivalent to 1.5 × 10$^{-5}$ U/ml, C and D) at 37°C for 20 min or 6 h, respectively. The inhibition experiments were carried out in the presence of 1 mM ZPP in the same conditions (B and D). The metabolic reactions were stopped by the addition of 2% phosphoric acid. After centrifugation, the supernatants were analyzed by HPLC.

Fig. 9. Enzymatic conversion rate of C-AVP$_{4-9}$ to AVP$_{4-9}$. Various concentrations of C-AVP$_{4-9}$ were incubated with authentic PPCE (0.1 U/ml, A) or mouse cerebral homogenate (20 mg cerebrum/ml equivalent to 1.5 × 10$^{-5}$ U/ml, B) at 37°C for 1 or 30 min, respectively. The metabolic reactions were stopped by the addition of 2% phosphoric acid. After centrifugation, the supernatants were analyzed by HPLC. Each point represents the mean ± S.E. of three separate experiments.

(Table 2). The $K_{p,app}$ values of $^{35}$S-labeled AVP$_{4-9}$ and $^{125}$I-labeled C-AVP$_{4-9}$ in the parenchyma fraction were markedly higher than the plasma volume in the parenchyma fraction and $K_{p,app}$ values of each labeled peptide in the capillary fractions (Table 2), suggesting that almost all of these labeled peptides are distributed to the parenchyma in the whole cerebrum. Therefore, the $K_{p,app}$ values of $^{35}$S-labeled AVP$_{4-9}$ and $^{125}$I-labeled C-AVP$_{4-9}$ in the whole cerebrum demonstrated in Fig. 4 nearly indicate the $K_{p,app}$ value of these labeled peptides in the cerebral parenchyma, and we evaluated the BBB permeation clearances of $^{35}$S-labeled AVP$_{4-9}$ and $^{125}$I-labeled C-AVP$_{4-9}$ with the $K_{p,app}$ values shown in Fig. 4 by the integration plot method (Smith et al., 1987; Kakae et al., 1996; Yang et al., 1997). With the integration plotting, the BBB permeation clearances of $^{35}$S-labeled AVP$_{4-9}$ and $^{125}$I-labeled C-AVP$_{4-9}$ were estimated to be 1.47 ± 0.09 × 10$^{-4}$ and 3.10 ± 0.35 × 10$^{-4}$ ml/min/g cerebrum, respectively (Fig. 5), suggesting that C-AVP$_{4-9}$ is transported through the BBB to the cerebral parenchyma more effectively than AVP$_{4-9}$.

Monolayers of MBEC4 cells were used in in vitro internalization studies. Because the acid wash treatment removes the labeled peptides binding to the cell surface, the acid-resistant binding of the labeled peptides expresses the internalized labeled peptides into the cells (Terasaki et al., 1989, 1992). The slight time dependence of acid-resistant bindings of extracellular space marker, $[^3$H]inulin, coexisting with $^{35}$S-labeled AVP$_{4-9}$ or $^{125}$I-labeled C-AVP$_{4-9}$, to MBEC4 (Fig. 6) may be ascribed to the fluid-phase endocytosis of MBEC4 (Terasaki et al., 1992). From 10 min after the incubation, the acid-resistant binding of $^{125}$I-labeled C-AVP$_{4-9}$ was higher than that of $^{35}$S-labeled AVP$_{4-9}$ (Fig. 6), suggesting that C-AVP$_{4-9}$ is internalized into the brain capillary endothelial cells more effectively than AVP$_{4-9}$. The $K_0$ values of RME-transported peptides reported previously were as follows: atrial natriuretic factor (400 mU, using cultured bovine brain capillary endothelial cells; Smith et al., 1988), transferrin (5.6 nM, isolated human brain capillaries; Partridge et al., 1987), insulin (2.9 nM, isolated human brain capillaries; Frank et al., 1986), and leptin (5.1 nM, isolated human brain capillaries; Golden et al., 1997). Although the $K_D$ values of AVP$_{4-9}$ for the low-affinity sites (45.7 μM) and of C-AVP$_{4-9}$ (16.4 μM) were in good agreement with the values of E-2078 (4.62 μM, using isolated bovine brain capillaries; Terasaki et al., 1989), ebiratide (15.9 μM, cultured bovine brain capillary endothelial cells; Terasaki et al., 1992; 62.1 μM, isolated bovine brain capillaries, Shimura et al., 1991), and histone (15.2 μM, isolated bovine brain capillaries, Partridge et al., 1989), which are internalized into the BBB via AME. In light of the agreements of $K_D$ values of AVP$_{4-9}$ (low affinity) and C-AVP$_{4-9}$ with the values of AME-transported peptides in the previous reports and the basicities of both peptides, it is suggested that C-AVP$_{4-9}$ and a part of AVP$_{4-9}$ are internalized into the BBB via AME.

To more precisely elucidate the mechanism underlying the internalization of C-AVP$_{4-9}$ into the BBB, we examined the acid-resistant binding of $^{125}$I-labeled C-AVP$_{4-9}$ in several conditions. The osmolarity dependence of the acid-resistant binding of $^{125}$I-labeled C-AVP$_{4-9}$ to MBEC4 (Table 3) suggests that C-AVP$_{4-9}$ is significantly internalized into MBEC4. As shown in Table 3, the acid-resistant binding of $^{125}$I-labeled C-AVP$_{4-9}$ to MBEC4 was significantly inhibited by lower temperature, dancyldcaderine (an endocytosis inhibitor) and a suppressor of the coated pit formation; Haigler
et al., 1980), phenylarsine oxide (an endocytosis inhibitor and a denaturant of the SH group in the cell membrane; Knutson et al., 1983), and 2,4-dinitrophenol (a metabolic inhibitor and an uncoupler of phosphorylation). In addition, as shown in Table 4, the cationic peptides [poly(l-lysine), protamine, and AVP₄₋₉] significantly inhibited the acid-resistant binding of ¹²⁵I-labeled C-AVP₄₋₉ to MBEC4, whereas an anionic peptide [poly(l-glutamic acid)] and the V₁ and V₂ vasopressin receptor antagonists had no effect on the acid-resistant binding. These results suggest that C-AVP₄₋₉ is internalized into the BBB via the energy-dependent AME by its basicity, independently of the vasopressin receptors.

We also examined the conversion of C-AVP₄₋₉ to AVP₄₋₉ in the cerebrum in vitro. The conversions of C-AVP₄₋₉ by the authentic PPCE and the mouse cerebral homogenate were inhibited by the PPCE-specific inhibitor ZPP (Fig. 8), and the authentic PPCE and the mouse cerebral homogenate agreed well with the value by the authentic PPCE (Fig. 9), suggesting that C-AVP₄₋₉ is converted to AVP₄₋₉ by PPCE in the cerebrum.

In conclusion, the results of our studies suggest that C-AVP₄₋₉ is transported through the BBB via AME to the cerebral parenchyma more effectively than its parent peptide, AVP₄₋₉. The results also suggest that C-AVP₄₋₉ is converted to AVP₄₋₉ in the cerebrum, consistent with our molecular design concept of C-AVP₄₋₉. Chemical modification to obtain a peptide with basicity and convertibility to the active form in the CNS is a promising strategy for the conversion of native physiologically active peptides into neuropharmaceuticals.

Acknowledgments

We thank Drs. Hiroshi Nagata and Ken-ichi Hosoya for their valuable discussions, and Dr. Wei-Xing Yang for the expert guidance and assistance in the capillary depletion study.

References


