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Kinetics of arginine-vasopressin uptake at the blood-brain barrier

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Uptake of arginine-vasopressin, VP, at the luminal side of the blood-brain barrier (BBB) was studied by means of an in situ brain perfusion technique in the guinea-pig. Kinetic experiments revealed a saturable peptide influx into the parietal cortex, caudate nucleus and hippocampus with K_m between 2.1 and 2.7 μM, and V_{max} ranging from 4.9 to 5.6 pmol · min^{-1} · g^{-1}. The non-saturable component, K_d, was not significantly different from zero. Influx of VP into the brain was not altered by the presence of the peptide fragments: VP-(1–8), pressinoic acid and [pGlu^4,Cyt^6]VP-(4–9) at 4.5 μM, nor yet by the aminopeptidase inhibitor, bestatin (0.5 mM) and the L-amino acid transport system substrates, L-tyrosine and L-phenylalanine at 5 mM. At a perfusate concentration of 4.5 μM, the V_1-vasopressinergic receptor antagonist, d(CH$_2$)$_5$[Tyr(Me)^2]VP, reduced VP influx; regional K_i values, assuming that the observed inhibitions were purely competitive, ranged between 4.7 and 8.5 μM. It is concluded that there is an apparent cerebrovascular permeability to circulating VP due to the presence of a carrier-mediated transport system for the peptide located at the luminal side. The mechanism for VP BBB uptake exhibits no affinity for peptide fragments and large neutral amino acids, but requires reception of the intact molecule, which may be the same initial step for both the BBB VP transporter and the V_1-receptor.

Introduction

Arginine-vasopressin, VP, is an important regulatory peptide for a number of peripheral and central functions [1]. Both circulating and central VP may affect higher integrative processes of the brain, including memory, attention, and avoidance behaviour [2–5]. The peptide and its fragments have been also studied for possible therapeutic applications in memory disorders caused by brain trauma, senile dementia and Alzheimer's disease [6,7]. Evidence suggests that VP is involved in the regulation of brain water metabolism [8], as well as in the pathogenesis of brain edema [9].

Although circulating VP does exert certain central effects, there has been controversy regarding the permeability of the blood-brain barrier (BBB) to this peptide. Earlier in vivo work suggested total impermeability of the BBB to VP, with independent regulation of peripheral and central pools [10,11]. However, more recent work indicates the presence of a carrier responsible for the transport of this peptide from cerebrospinal fluid (CSF) and brain to blood [12]. In vitro studies have demonstrated only symmetrical diffusion of this peptide or its fragments across cerebrovascular endothelial monolayers with no evidence for carrier mediation [13]. Additional studies though have found receptors for VP in isolated cerebral microvessels [14], and the saturable retention of the peptide in some brain regions in vivo [15]. In order to further clarify what appears to be conflicting data, kinetic analysis and characterization of the cellular uptake of blood-borne VP at the luminal side of the BBB was investigated using an in situ long-term brain perfusion technique in the guinea-pig [16]. This method has been successfully applied to characterize transport, receptor and enzymatic events for a number of slowly-penetrating substances at the BBB including peptides [17–21]. The present work has demonstrated an apparent cerebrovascular permeability to VP due to the presence of

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carrier-mediated transport mechanism located at the luminal side.

Material and Methods

Chemicals, peptides and radioactive molecules. VP, VP-(1–8), d(CH_2)$_5$[Tyr(Me)^2]VP, pressinoic acid, [pGlu^4,Cyt^6]VP-(4–9), L-phenylalanine, L-tyrosine and bestatin were obtained from Sigma (Poole, U.K. and St. Louis, MO) and Peninsula (Belmont, CA). BCH was purchased from Calbiochem. (Boehringer, F.G.R.). [[3,4,5(n)-^{3}H][Phenylalanyl-VP, 68.5 Ci/nmol and D-[^{14}C]mannitol were purchased from New England Nuclear (Boston, MA). Radiochemical purity of labelled vasopressin was established prior to use by thin-layer chromatography on silica gel using the following solvent system: *n*-butanol/ pyridine/ acetic acid/water (30 : 20 : 6 : 24, v/v), and by high pressure liquid chromatography on a Zorbax Bio Series C8 eluting isocratically with 0.2% TFA/acetonitrile (85 : 15, v/v). The chromatography failed to reveal degradation products of the peptide or free radioactive L-[^{3}H]-phenylalanine (labelled residue of peptide) in the perfusion fluid (arterial inflow), and indicated a purity of about 99%.

Perfusion technique for the guinea-pig brain. Vascular perfusion of the ipsilateral guinea-pig forebrain in situ has recently been described in detail [16] and here will only be briefly summarized. Adult guinea-pigs of either sex weighing 250 to 350 g were anesthetized with thiopentone sodium (30–35 mg/kg) prior to surgical exposure of the neck vessels. The right common carotid artery was cannulated with a fine polythene tubing connected to the extracorporeal perfusion circuit. Immediately after the start of perfusion, the contralateral carotid artery was ligated and both jugular veins cut to allow free drainage of the perfusate. The perfusion medium consisted of washed sheep red cells suspended in a saline medium of a composition previously reported. The hematocrit of the perfusion fluid was 20% and the colloid osmotic pressure maintained at normal physiologic level by addition of dextran (M_r 70000) at a concentration of 48 g · l^{-1}. The perfusion medium was delivered to the brain by means of a peristaltic pump with a perfusion pressure kept slightly above the animal's own blood pressure (15 to 20 mmHg) to eliminate any ingress from the systemic circulation. The uniqe anatomy of the cerebral circulation in the guinea-pig [21] ensured the functional separation between artificial and vertebral circulation previously confirmed with isotope experiments [18].

Isotopically labelled ^{3}H-VP in the presence of varying concentrations of unalbelled VP, ranging from 0.003 to 10 μM, was introduced into the perfusion circuit by a slow-drive syringe. In order to distinguish luminal BBB uptake of the intact tracer from its peptide fragments and/or competitors, the kinetics of entry of ^{3}H-VP were studied in the presesnce of VP-(1–8), pressinoic acid, [pGlu^4,Cyt^6]VP-(4–9), 2-aminobicyclo-[2.2.1]heptane-2-carboxylic acid (BCH), L-phenylalanine (amino acid-labelled residue of VP), and L-tyrosine, at concentration of 4.5 μM and 5 mM, as well as in the presence of the most potent aminopeptidase inhibitor, bestatin (0.5 mM). In a separate series of experiments, the effect of V_1-vasopressinergic receptor antagonist, 1-β-mercapto-β,βcyclopentamethylenepropionic acid,2-(*O*-methyl)tyrosine VP, (d(CH_2)$_5$[Tyr(Me)^2]VP), at perfusate concentrations of 2.7 and 4.5 μM, on the kinetic entry of ^{3}H-VP into the perfused, ipsilateral forebrain were studied. At given times, ranging from 0.5 to 20 min, the perfusion was terminated by severing the right common carotid artery and decapitating the animal. The brain was then removed and dissected into several regions for scintillation counting.

Counting of radioactive samples. The ipsilateral perfused forebrain was divided into the following regions: parietal cortex, caudate nucleus, and hippocampus. Brain samples of 80 to 100 mg wet weight were dispensed into pre-weighed scintillation vials and solubilized overnight in 0.5 ml Soluene (Packard) or BTS-450 (Beckman). The perfusion medium was centrifuged and 25 μl samples of supernatant were prepared in the same way by addition of about 100 mg of non-radioactive brain. Before counting, the samples were treated with 4 ml of scintillant (Beckman Ready Organic). ^{3}H radioactivity was determined on the LKB Spectral Beta scintillation spectrometer or Beckman LS7500 liquid scintillation counter.

Calculations. The permeability of the BBB to ^{3}H-VP was expressed by the unidirectional transfer constant, K_{in} determined from multiple time-point/graphic analysis as previously reported [22–25,16] by employing the equation:

$$\frac{C_{BR}(T)}{C_{PL}(T)} = K_{in}\frac{\int_0^T C_{PL}(t)\,dt}{C_{PL}(T)} + V_i \qquad (1)$$

where $C_{BR}(T)$ and $C_{PL}(T)$ are radioactivities of ^{3}H-VP measured per unit mass of brain and plasma, respectively, at time T; T is the time of perfusion. Eqn. 1 defines a straight line with a slope K_{in} (the unidirectional transfer constant) and ordinate intercept V_i, that includes the possibility of an initial distribution of peptide in a rapidly reversible compartment(s); vascular compartment, the BBB and/or compartments in parallel with the BBB. Since during vascular brain perfusion state, the radioactivity of the test solute remains constant in the arterial inflow, Eqn. 1 becomes:

$$\frac{C_{BR}(T)}{C_{PL}(T)} = K_{in}T + V_i \qquad (2)$$

as previously described [16,20]. Departure of experi-

mental points from linearity indicates backflux of the solute from the brain to blood. The K_{in} value was also determined by means of single time-point analysis using the equation as previously developed [26,27] and applied to the brain perfusion data [16]:

$$K_{in} = \frac{C_{BR}(T)}{\int_0^T C_{PL}(t)\,dt} \tag{3}$$

Since radioactivity of the test-solute remains constant in the arterial inflow, Eqn. 3 becomes:

$$K_{in} = \frac{C_{BR}(T)}{C_{PL}T} \tag{4}$$

Correction for the residual vascular radioactivity of tracer in single time-point uptake studies was made by washing the intravascular tracer from the brain blood vessels by perfusing the brain for 1 min with tracer-free fluid [28]. In addition, in a separate series of experiments, uptake of intravascular brain marker, D-[^{14}C]mannitol, was measured using the same procedure.

Experimentally determined unidirectional transfer constant, K_{in}, represents the cerebrovascular permeability surface area product, PA, [28–30] and is given in the same units ($ml \cdot min^{-1} \cdot g^{-1}$). In the case when ^{3}H-VP was the only studied test-solute, with varying concentrations of unlabelled VP included, K_{in} for VP during the vascular brain perfusion is defined as:

$$PA = K_{in} = \frac{V_{max}}{(K_m + C_{cap})} + K_d \tag{5}$$

where V_{max} is the maximal influx rate of the saturable component, K_m is the half-saturation constant, and K_d is the constant for non-saturable diffusion; C_{cap} is the mean capillary concentration of peptide. Eqn. 5 has been previously developed for the blood-brain uptake studies [31] and to characterize amino acid transport [32,33] as well as peptide interaction during artificial blood perfusion of the guinea-pig brain, over longer time intervals [30]. Eqn. 5 can be further simplified as previously suggested [34,35,30] to:

$$K_{in} = \frac{V_{max}}{K_m + C_{PL}} + K_d \tag{6}$$

since during perfusion conditions, the cerebral blood flow, F, measured in separate sets of animals was about 1 $ml \cdot min^{-1} \cdot g^{-1}$ for the ipsilateral perfused forebrain, which is much greater than the highest measured K_{in}, i.e. $F \gg K_{in}$, and the difference between C_{cap} and concentration of peptide in the arterial inflow, C_{PL}, becomes negligible.

Best fit values for V_{max}, K_m and K_d were obtained for VP by fitting Eqn. 6 to the brain vascular perfusion data with weighted non-linear least squares [36–38] as previously reported for amino acid brain perfusion [35], and for peptide brain perfusion [30].

Values are expressed as means ± S.E., and estimates of S.E. were obtained by partitioning the residual sum of squares from the fit of the total data set.

Unidirectional VP influx, J_{in}, into the brain was calculated as:

$$J_{in} = F(1 - e^{-K_{in}/F})C_{PL} \tag{7}$$

and since $F \gg K_{in}$ Eqn. 7 approximates [32] to:

$$J_{in} \approx K_{in}C_{PL} \tag{8}$$

Unidirectional influx of VP is related to the kinetic constants, K_m, V_{max} and K_d by the following equation:

$$J_{in} = \frac{V_{max}C_{PL}}{(K_m + C_{PL})} + K_dC_{PL} \tag{9}$$

Values for V_{max}, K_m and K_d are means ± S.E. for 27 animals. Analysis of variance was used to compare the individual means. Based on the inhibition data, the inhibitor constants, K_i for the saturable component, were estimated by means of 'velocity ratio' [39,40] in which the uninhibited rate of influx, J_{in} may be related to the inhibited rate of influx, J_i, assuming that observed inhibitions were purely competitive. Thus, saturable influx in the presence of the inhibitor may be determined using the following equation [39]:

$$J_i = \frac{V_{max}C_{PL}}{K_m(1 + C_i/K_i) + C_{PL}} \tag{10}$$

where J_i is influx of VP at perfusate concentration C_{PL}, in the presence of the perfusate concentration of the inhibitor, C_i. Thus from the 'velocity ratio' of the saturable component of VP influx J_{in} is related to J_i as [39,40]:

$$\frac{J_{in}}{J_i} = \frac{K_m(1 + C_i/K_i) + C_{PL}}{K_m + C_{PL}} \tag{11}$$

from where the inhibitor constant, K_i, may be calculated as:

$$K_i = \frac{J_iK_mC_i}{(J_{in} - J_i)(K_m + C_{PL})} \tag{12}$$

Values for K_i are means ± S.E., obtained from 3 to 8 experiments with VP influx assessed at a perfusate concentration of 3 nM in the absence and presence of a single inhibitor at 4.5 μM concentration.

In comparing K_{in} and V_i parameters where variances between the two groups, in the absence and presence of inhibitor, did not significantly differ by the Bartlett test for homogeneity of variance, the two-sample t-test for

samples with equal variance was applied. When variance did differ significantly, Satterwaite's method [41] was used. Multiplicity was dealt with through the Bonferroni adjustment and $p < 0.05$ was chosen as the experimental significance level.

Results

Fig. 1 illustrates multiple time-brain uptake plot obtained during perfusion of the parietal cortex with ^{3}H-VP in the absence and presence of 10 μM unlabelled peptide. There is a time-dependent progressive uptake of labeled peptide, giving C_{BR}/C_{PL} values of about 0.6% at the first minute of perfusion to about 6% at 20 min; strong self-inhibitory effect is apparent (inhibition of the slope by 83%), and similar results were obtained for hippocampus and caudate nucleus (data not shown). Table I summarizes regional values for the unidirectional transfer constant, K_{in}, and initial volume of distribution, V_i, for ^{3}H-VP obtained by multiple time-point/graphic analysis, and the vlaues for K_{in} obtained in a separate series of experiments using single time-point analysis (10 min perfusion with labelled peptide followed by 1 min perfusion with tracer-free medium). It can be seen that regional K_{in} values computed by either type of analysis differ only by 4 to 5% which was insignificant by analysis of variance. Regional V_i values computed by multiple time-point analysis were similar and differences were insignificant by analysis of variance; the single time-point method involved an automatic correction for V_i values, or residual vascular radioactivity. However, regional differences in K_{in} values between cortex, caudate nucleus and hippocampus were statistically significant by analysis of variance. In addition, K_{in} values for D-[^{14}C]mannitol (extracellular reference) obtained in a separate series of experiments by single time-point procedure (10 min perfusion with labelled mannitol followed by 1 min perfusion with tracer-free medium) were given in Table I; values were comparable with K_{in} values for D-mannitol computed by multiple time-point/graphic analysis in the guinea-pig, using the same perfusion technique, as previously reported (Table II, Ref. 19). It can be seen that the permeability constant, P, computed for VP is about one order of magnitude higher than for mannitol. Olive oil/artificial plasma, partition coefficients (PC) for VP and D-mannitol were 0.057 and 0.0028, respectively.

Fig. 2 illustrates multiple time-brain uptake plot obtained during perfusion of hippocampus with ^{3}H-VP in

TABLE I

Capillary undirectional constant, K_{in} and initial volume of distribution, V_i, of ^{3}H-VP in the perfused guinea-pig brain

Multiple time-point/graphic [a] and single time-point [b] analysis were used to estimate K_{in} and V_i parameters. K_{in} values for D-[^{14}C]mannitol obtained in a separate series or experiments by single time-points analysis [b] were given for comparison. Cerebrovascular permeability constant, P, is determined on the assumption of a capillary surface area of 100 cm$^2 \cdot$g^{-1} brain [57]. Values are means ± S.E. for 3–10 animals.

Brain region	K_{in} (μl·min^{-1}·g^{-1})	V_i (ml·100 g^{-1})	P (nm·s^{-1})
^{3}H-VP			
Parietal cortex	2.78 ± 0.04 [a]	0.27 ± 0.04	4.63
	2.89 ± 0.21 [b]	–	4.81
Hippocampus	1.95 ± 0.03 [a]	0.35 ± 0.03	3.25
	2.05 ± 0.22 [b]	–	3.42
Caudate nucleus	2.24 ± 0.03 [a]	0.25 ± 0.03	3.73
	2.37 ± 0.17 [b]	–	3.95
D-[^{14}C]Mannitol			
Parietal cortex	0.29 ± 0.02 [b]	–	0.48
Hippocampus	0.26 ± 0.05 [b]	–	0.43
Caudate nucleus	0.22 ± 0.04 [b]	–	0.36

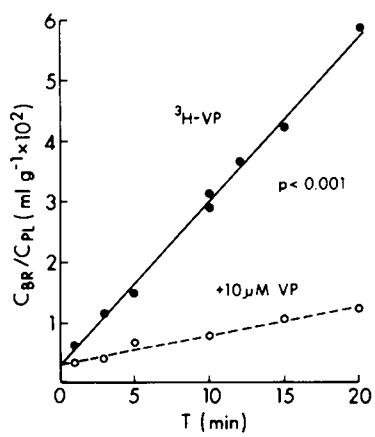

Fig. 1. Kinetics of entry of ^{3}H-VP into the parietal cortex of the perfused guinea-pig brain in the absence (solid points and line) and presence of 10 μM unlabelled VP (open points and dashed line). ^{3}H radioactivity (dpm·g^{-1} of brain/dpm·ml^{-1} of plasma perfusate) is plotted against the perfusion time, T. Each point represents a single experiment; K_{in} values were graphically estimated as slopes of linear best regression lines, from the multiple-time series, as compared by analysis of variance (p).

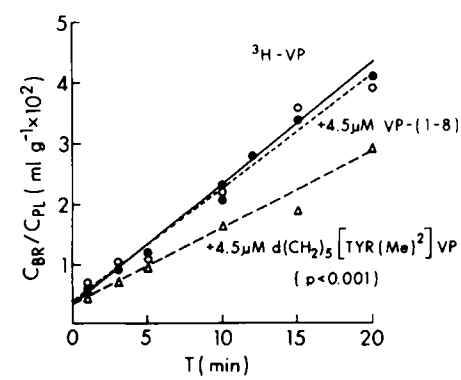

Fig. 2. Kinetics of entry of ^{3}H-VP into the hippocampus of the perfused guinea-pig brain in the absence (solid points and line) and presence of 4.5 μM unlabelled VP-(1–8) (open points and dashed line) and d(CH$_2$)$_5$[Tyr(Me)^2]VP (triangles and interrupted line). Other explanation as for Fig. 1.

TABLE II

Inhibition of VP uptake at the luminal side of the BBB in the perfused guinea-pig brain

Unidirectional influx of VP was measured at perfusate concentration of 3 nM in the absence (J_{in}) and presence (J_i) of unlabelled inhibitor at 4.5 μM. The percentage change in VP influx was calculated from $(1-(J_i/J_{in}))\times 100$. When brain was perfused with unlabelled peptide fragments, VP-(1–8) (n = 6), pressinoic acid (n = 8) and [pGlu^4,Cyt^6]VP-(4–9) (n = 7), and with aminopeptidase inhibitor, bestatin (0.5 mM, n = 5) or with L-amino acid transport system substrates, BCH (n = 5 to 6), L-tyrosine (n = 5) and L-phenylalanine (n = 5), % changes in VP influx were within range of ±4 to 7%, and were not significantly different in comparison to the corresponding regional control values. Values given are means ± S.E. for 3–6 animals.

Inhibitor (4.5 μM)	Brain region	% inhibition in VP influx
VP	parietal cortex	65 ± 2
	caudate nucleus	54 ± 3
	hippocampus	57 ± 4
d(CH$_2$)5[Tyr(Me)^2]VP	parietal cortex	49 ± 2
	caudate nucleus	41 ± 2
	hippocampus	35 ± 2

the absence and presence of 4.5 μM VP-(1–8) and V_1-vasopressinergic receptor antagonist, d(CH$_2$)$_5$[Tyr(Me)^2]VP. It can be seen that VP-(1–8) was without effect on ^{3}H-VP kinetics of entry, and similar results were obtained for the cortex and caudate nucleus. On the other hand d(CH$_2$)$_5$[Tyr(Me)^2]VP applied at the same concentration caused a significant reduction in the BBB uptake of ^{3}H-VP. Effects of different potential inhibitors on unidirectional influx of VP measured at perfusate concentration of 3 nM in the presence of unlabelled inhibitor at 4.5 μM are given in Table II. Since in the presence of peptide fragments, VP-(1–8), pressinoic acid (1–6) and [pGlu^4,Cyt^6]VP-(4–9) and the aminopeptidase inhibitor, bestatin (0.5 mM), no significant alterations in VP influx were observed, (changes were only within a range of ±4 to 7%), individual regional values were not presented in Table II. Similarly, in the presence of L-amino acid transport system substrates, BCH, L-tyrosine and L-phenylalanine, both at concentrations of 4.5 μM and 5 mM no significant changes in VP influx could be demonstrated and individual regional values were not presented in Table II. However, it can be seen that 4.5 μM of V_1-receptor antagonist, d(CH$_2$)$_5$[Tyr(Me)^2]VP, caused a significant inhibition in VP influx ranging from 35 to 49%, in the hippocampus, caudate nucleus and parietal cortex; this inhibition was less potent than inhibition produced by equimolar concentration of peptide itself, which ranged between 54 and 69% (Table II). In eight separate experiments single time-point analysis was applied to test effects of 2.7 μM d(CH$_2$)$_5$[Tyr(Me)^2]VP on the kinetics of entry of ^{3}H-VP, and regional (mean ± S.E.) K_{in} values ($\mu l \cdot min^{-1} \cdot g^{-1}$) for parietal cortex, hippocampus and caudate nucleus were 2.09 ± 0.18, 1.67 ± 0.17 and 1.94 ± 0.19, respectively; these values were significantly lower in comparison to control values in the absence of potential inhibitor.

Fig. 3 illustrates the relationship between the unidirectional transfer constant K_{in} for ^{3}H-VP in the parietal cortex and concentration of unlabelled peptide(1–9) in the perfusion medium. The curve represents the best fit of Eqn. 5 to the experimentally obtained K_{in} values using weighted non-linear least squares. It can be seen there is a concentration dependence of K_{in} for ^{3}H-VP that follows Michaelis-Menten kinetics. Summarized values for kinetic parameters, K_m and V_{max}, for the saturable component and K_d, for the non-saturable component are given in Table III. There were no statistical regional differences in any of the estimated kinetic parameters: K_m ranged from 2.1 to 2.7 μM, and V_{max} between 5 and 5.5 $pmol \cdot min^{-1} \cdot g^{-1}$. K_d values were insignificant in magnitude and not different from zero ($P > 0.05$), suggesting that all available VP exposed to the luminal side of the BBB is taken up by a saturable mechanism. Fig. 4 shows the relationship between unidirectional VP influx into the parietal

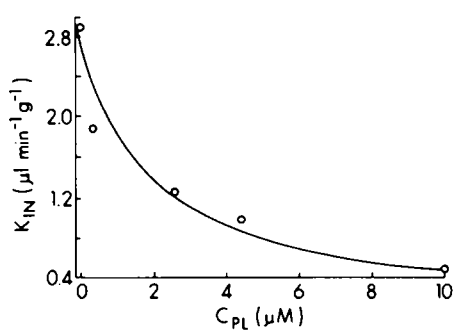

Fig. 3. Capillary unidirectional constant (K_{in}) for ^{3}H-VP in the parietal cortex plotted against VP concentration in the perfusate, C_{PL}. The curve is non-linear regression weighted least squares fit of K_{in} values to Eqn. 6; each point represents the mean value for 3–10 animals.

TABLE III

Kinetic parameters K_m, V_{max} and K_d, for VP cerebrovascular uptake during a vascular perfusion of the guinea-pig brain

Values are means ± S.E. for five K_{in} observations obtained by either multiple time-point/graphic and/or single time-point analysis, based on 27 individual experiments. K_{in} values for each brain region are used to estimate best-fit values for K_m, V_{max}, and K_d by fitting Eqn. 6 to the brain vascular perfusion data with weighted non-linear least squares.

	K_m (μM)	V_{max} ($pmol \cdot min^{-1} \cdot g^{-1}$)	K_d ($\mu l \cdot min^{-1} \cdot g^{-1}$)
Parietal cortex	2.08 ± 0.32	5.49 ± 0.74	0.021 ± 0.044
Caudate nucleus	2.61 ± 0.48	5.56 ± 0.89	0.00 ± 0.055
Hippocampus	2.67 ± 0.31	4.92 ± 0.50	0.00 ± 0.062

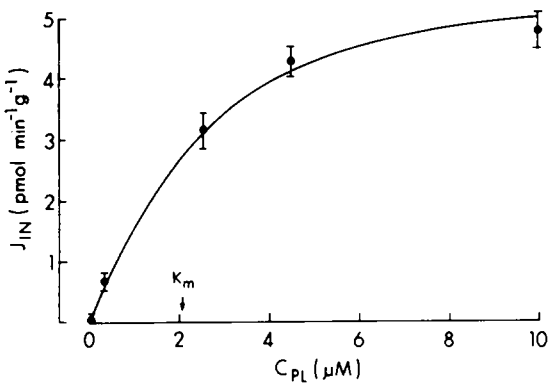

Fig. 4. VP influx, J_{in}, into the parietal cortex is plotted against VP concentration in the perfusate, C_{PL}. The curve represents the VP influx predicted by Eqns. 6 and 9; each point represents the mean ± S.E. for 3–10 animals.

cortex and concentration of unlabelled peptide, C_{PL}, in the perfusion medium. J_{in} values were calculated from K_{in} and C_{PL} values using Eqn. 8; an increase of the unidirectional influx of VP as a saturable function of VP concentration in the perfusate is apparent. Based on the inhibition data, respective inhibitor constants, K_i, were estimated by means of a 'velocity ratio', assuming that the observed inhibitions were competitive. Table IV shows that VP itself was the most effective inhibitor, while d(CH$_2$)$_5$[Tyr(Me)^2]VP produced significantly higher K_i values and the same was true for the K_i/K_m ratio. All other tested compounds including peptide fragments and amino acids did not produce any significant changes in VP influx, and K_i values tended to infinity.

No significant changes in perfusion pressure were observed after introduction of either radiolabelled VP alone or in the presence of different concentrations of unlabelled peptide, peptide fragments, L-amino acids, bestatin, and V_1-receptor antagonist.

TABLE IV

Kinetic inhibition of VP influx in the perfused guinea-pig brain brain

VP influx was assessed at a perfusate concentration of 3 nM in the absence and presence of a single inhibitor at 4.5 μM concentration. The estimate of K_i was based on regional K_m values of 2.08 to 2.76 μM measured for VP (see Table III), and was calculated using a velocity ratio Eqn. 12 [39,40] in which the uninhibited rate of influx may be related to the inhibited rate provided the inhibition is competitive. When brains were perfused with VP-(1–8), pressinoic acid, [pGlu^4,Cyt^6]VP-(4–9), BCH, L-tyrosine, and L-phenylalanine, no significant changes in any of the studied regions were observed and K_i tended to infinity. Values are means ± S.E. for 3–8 animals.

	K_i (μM)	K_i/K_m
VP (Arg)		
Parietal cortex	2.44 ± 0.09	1.17 ± 0.26
Caudate nucleus	3.75 ± 0.17	1.43 ± 0.40
Hippocampus	3.36 ± 0.36	1.25 ± 0.32
d(CH$_2$)$_5$[Tyr(Me)^2]VP		
Parietal cortex	4.69 ± 0.09	2.25 ± 0.36
Caudate nucleus	6.33 ± 0.03	2.42 ± 0.56
Hippocampus	8.52 ± 0.83	3.19 ± 0.77
VP-(1–8)	∞	–
Pressinoic acid	∞	–
[pGlu^4,Cyt^6]VP-(4–9)	∞	–
BCH	∞	–
L-Tyrosine	∞	–
L-Phenylalanine	∞	–

Discussion

The present study provides the first direct evidence for the presence of a saturable cellular uptake mechanism for circulating VP located at the luminal side of the BBB in the perfused guinea-pig brain. Kinetic analysis indicated that uptake system for VP at the luminal surface of the BBB is of very low capacity (5–5.5 pmol · min^{-1} · g^{-1}), and as a result of relatively large K_m (Table III), the unidirectional influx of VP from the artificial blood can be regarded as a linear function of VP concentration over the range between 0 to about 300 nM (Fig. 4). Taking into account that normal plasma levels of VP are also very low, in the guinea-pig less than 50 fmol/l [42], such a system may be of physiological importance for VP homeostasis in the brain.

Competition experiments, when the kinetics of entry of ^{3}H-VP were studied in the presence of inhibitory concentrations of the peptide itself, peptide fragments, large neutral amino acids and aminopeptidase inhibitor (Fig. 2, Tables II and IV), suggest that circulating VP is taken up in an intact form at the BBB, using a carrier-mediated mechanism which does not have any affinity for peptide fragments and/or large neutral L-amino acids. In addition, it has been demonstrated that the peptide cannot use the large neutral L-amino acid BBB transporter, although it is known that VP may affect the passage of several substance across the BBB, including large neutral amino acids [10,15]. However, the fact that VP may be taken up intact at the luminal side of the BBB, does not rule out the possibility that it may be metabolized subsequently in one of the compartment(s) in parallel such as: cytosolic endothelial space, abluminal surface of the BBB, glial-end foot layer in apposition with the abluminal side of the capillary endothelium, and/or vasopressinergic synaptic regions juxtaposed to the brain microvessels [1]. Metabolic degradation of peptide by the synaptic membranes and behavioral experiments with peptide fragments [43] have suggested that the VP molecule may be in fact only the precursor for more potent physiologically active neuropeptides that are formed by proteolytic fragmentations of VP, predominantly by aminopeptidases, in the brain [44]. Recent work has confirmed that exogenous VP is rapidly metabolized in vivo mainly by aminopeptidase activity, suggesting that behavioral effects seen after

microinjection of the peptide into the brain may be partially due to the generation of its potent fragments [45]. Present experiments indicate that transformation of circulating VP to a highly active [pGlu^4,Cyt^6]VP-(4–9) [43], as well as into 1–6 and 1–8 fragments, pressinoic acid and VP-(1–8), respectively (Table IV), does not take place at the first step, i.e., at the luminal interface of the BBB, since these peptide fragments were ineffective in inhibiting ^{3}H-VP kinetics of entry (Table II). Also, L-phenylalanine (labelled amino acid residue of VP), as well as aminopeptidase inhibitor, bestatin (Table II and IV) did not affect uptake of ^{3}H-VP, suggesting there was no proteolytic fragmentation of VP with liberation of free labelled amino acid residue, L-[^{3}H]phenylalanine. However, the kinetic features of the VP BBB transport system and estimated cerebrovascular permeability constant, P, of 3.2 to 4.8 $nm \cdot s^{-1}$ (Table I, Fig. 1), suggest availability of circulating intact VP to the brain. Thus, once it has been taken up by the brain, blood-borne VP may exert its central actions either intact or as a precursor for more potent neuropeptides [43–45]. Further chromatographic analysis of the nature of radioactivity in the brain should provide more information with respect to post-transport steps, i.e., a possible subsequent metabolic step of blood-borne VP in the brain, once it has been taken up by the luminal side of the BBB. The results of this study differ from earlier in vivo studies [10,11] which found no measurable brain extraction of peptide during 15s kinetic experiments, but may well be explained by the longer time of exposure, up to 20 min, since extremely short exposure time of peptide to the BBB in previous studies [10,11] may not be sufficient to characterize the BBB permeability to slowly penetrating compounds [16,20]. On the other hand, our results are in agreement with previous findings suggesting the presence of a carrier for VP from CSF to blood [12]. The present findings support the hypothesis that the correlation between CSF and plasma VP levels observed in previous studies [46,47], may be due to the penetration of peptide across the BBB.

Specific carrier-mediated transport systems at the luminal side of the BBB have been described for numerous classes of nutrients [48], thyroid hormones [49], and peptides, such as enkephalin-leucine and delta sleep-inducing peptide, at both the blood-CSF barrier [50,51] and BBB [17,19,30]. K_m values for VP determined in this study closely resemble those estimated for thyroid hormones [49], while the capacity of VP transport system is the lowest so far described. Uptake of several proteins, including insulin [52], IGF1 and 2 [53] and transferrin [54] has been demonstrated using isolated brain capillaries in vitro, with the half-saturation constants for these receptor-mediated transfers in the low nanomolar range. The results from the present study, also suggest that vasopressinergic endothelial receptors may be involved in VP BBB uptake (Fig. 2, Tables II and IV), since the influx of peptide was strongly inhibited in the presence of V_1-receptor antagonist, d(CH$_2$)$_5$[Tyr(Me)^2]VP. However, there is no direct evidence as to whether the V_1-receptor antagonist directly inhibits transport of circulating VP into the endothelial cells, as opposed to preventing only the entrapment of the peptide from the circulation [15]. Since K_m values for VP were in the low micromolar range (Table III), and since the apparent regional cerebrovascular permeability constants were estimated to be $P > 1$ $nm \cdot s^{-1}$ (Table I), our data may reflect carrier-mediated rather than receptor-mediated VP uptake [55]. However, it is possible that the reception of the intact VP molecule is required as an initial step for both the BBB VP transporter and V_1-receptor, as indicated by the structural similarity of VP and d(CH$_2$)$_5$[Tyr(Me)^2]VP [56]. Manipulations of the BBB with respect to the VP transport system may have important clinical implications in the treatment of memory disorders [6,7] as well as of brain edema resulting from a variety of pathological conditions [9].

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