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RAPID COMMUNICATION

Regional Variation in Transport of Pancreatic Polypeptide Across the Blood–Brain Barrier of Mice

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BANKS, W. A., A. J. KASTIN AND J. B. JASPAN. *Regional variation in transport of pancreatic polypeptide across the blood-brain barrier of mice.* PHARMACOL BIOCHEM BEHAV 51(1) 139–147, 1995.—Blood-borne pancreatic polypeptide (PP) affects pancreatic secretion indirectly by acting through the central nervous system (CNS). PP, which is apparently not synthesized by brain, must cross the blood-brain barrier (BBB) to reach areas such as the cerebellum, an area rich in PP receptors, and to account for the PP found in cerebrospinal fluid (CSF). We used multiple-time regression analysis to measure the unidirectional influx constant (K_i) into brain of intravenously injected radioiodinated PP (I-PP). The K_i was 1.15 (10^{-3}) ml/g · min and was inhibited by unlabeled PP but not by tyrosine. HPLC showed that radioactivity in the brain was mostly intact I-PP. Up to 0.065% of the injected dose entered each gram of brain with preferential entry into the cerebellum and the pons-medulla. Capillary depletion confirmed that intact I-PP penetrated the BBB. I-PP exited the brain by a nonsaturable process. These results show that I-PP crosses the BBB by a saturable system to reach its receptors behind the BBB.

Pancreas Brain-gut axis Cerebrospinal fluid Cerebellum

INTERACTIONS between the brain and the gastrointestinal tract have been a classic area of study. Although some of these interactions are mediated through neural connections, the basis for other interactions remains to be clarified (30). The discovery that peptides are found in both the gastrointestinal tract and in the central nervous system (CNS) has raised the possibility that a "gut-brain" axis may exist, although the nature of such axes may vary for specific peptides (27). Pancreatic polypeptide (PP) released into peripheral blood is thought to exert several effects through a direct action on the CNS. Effects postulated to be mediated through the CNS include inhibition of pancreatic exocrine secretions (50), control of feeding behavior (24), and release after hypoglycemia (28). The conjecture that blood-borne PP could directly affect the CNS is supported by the findings that although receptors specific for PP have been found in chickens, pigs, and dogs (2,3,33), the brain apparently does not express mRNA for PP (44). This suggests that the PP acting within the CNS must arise from a peripheral source.

Recent work has shown that blood-borne PP can reach the circumventricular organs (CVO) (51), small areas of the brain without a blood-brain barrier (BBB) (49). The CVOs, in turn, can affect many aspects of CNS function (35) through their neural connections with the rest of the brain. However, a portion of the BBB exists as an interface between the CVOs and the cerebrospinal fluid (CSF) (49) and between the CVOs and the adjacent neuropile behind the BBB (39,40). Therefore, substances entering the CVOs are blocked from reaching the CSF or the rest of the brain. If blood-borne PP is to reach PP receptors in such areas as the cerebellum (2,3), which does not contain CVOs, or to account for the PP found in CSF (34), then it must be able to cross the BBB.

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Several other peptides cross the BBB either by nonsaturable transmembrane diffusion (5,13) or by saturable mechanisms (7,23) in amounts sufficient to affect CNS function (9,38). We applied methods previously used to investigate those peptides to determine whether PP can cross the BBB.

METHOD

Measurement of Blood to Brain Entry Rates

Male ICR mice (Charles River Laboratory, Wilmington, MA) weighing 20–25 g and housed under 12L : 12D with food and water freely available were anesthetized with urethane (4 g/kg) IP. All studies were approved by the institution and strictly followed AAALAC guidelines. The right jugular vein and left carotid artery were exposed and mice received an intravenous (IV) injection into the jugular vein that contained 10^6 cpm of bovine [^{125}I]pancreatic polypeptide (I-PP) in a volume of 0.2 ml. The I-PP was a kind gift of Eli Lilly and Co. (Indianapolis, IN). It was shipped in 29.5% acetonitrile and ammonium acetate that was diluted 1 : 1 with a solution of chloride-free phosphate buffer solution with 1% bovine serum albumin before injection. The I-PP was used within 2 weeks of iodination. At timed intervals between 1 and 10 min after IV injection, the carotid artery was cut, arterial blood collected, the mouse decapitated, and the whole brain removed. The arterial blood was centrifuged at 4°C for 10 min at 4000 × *g* and the level of radioactivity in 0.05 ml of arterial serum was determined in a gamma counter. The log(percent of injection/ml of arterial serum) was expressed relative to time and the half-time disappearance from blood. Values for half-time disappearance from blood ($t_{1/2}$) and whole body volume of distribution (V_d) were computed from the slope and the intercept of this relationship (29).

The whole brain was dissected free of the pineal and pituitary, rinsed in 0.9% NaCl, blotted dry, and its level of radioactivity measured in a gamma counter. The rate of entry into the brain measured as the unidirectional influx constant, or K_i, and expressed in ml/g · min and the apparent volume of distribution, or V_i, in μl/g, were determined by multiple-time regression analysis (25,43) with the use of the equation

$$\text{Am/Cpt} = K_i[\int_0^t \text{Cp}(\tau)d\tau]/\text{Cpt} + V_i \quad (1)$$

where Am is cpm/g of brain, Cpt is cpm/ml of arterial serum, and exposure time is measured by the term $[\int_0^t \text{Cp}(\tau)d\tau]/\text{Cpt}$.

The percent of the IV injection present in a gram of brain (%Inj) was determined from the equation

$$\%\text{Inj} = K_i(\text{expt})(\text{Is}) \quad (2)$$

where expt is exposure time as determined above and Is is the percent of the injection found in 1 ml of arterial serum at that exposure time.

To test for a saturable transport process, other mice had unlabeled porcine PP, a kind gift of Eli Lilly and Co., at doses of 3.5 or 100 μg/mouse or the amino acid tyrosine at a dose of 3.5 μg/mouse (Sigma Chemical Co., St. Louis, MO) included in the IV injection (n = 8/group). To determine whether the K_i was constant throughout the whole brain, other mice (n = 10) had the cerebral cortex, cerebellum, and pons-medulla dissected from the rest of the brain. To simplify calculations by eliminating the vascular space, the brain was perfused free of blood by the severance of both jugular veins and perfusion of 20 ml of 0.9% NaCl through the left ventricle of the heart. Before the start of the perfusion, an arterial blood sample was obtained from the abdominal aorta and then the thoracic aorta was clamped so as to direct the perfusion cephalad. Multiple-time regression analysis was applied to these three regions, to the remainder of the brain, and, after addition of the levels of radioactivity and weights for each of these four regions, to whole brain.

High Performance Liquid Chromatography

Mice were injected with 10^6 cpm of I-PP IV and arterial blood was taken at 2, 5, and 20 min and whole brains were taken at 2, 5, and 10 min after injection. Different mice were used for each sample to expedite processing. Blood was collected in vials chilled in an ice water bath and immediately centrifuged at 3000 × *g* for 10 min at 4°C. Serum was lyophilized and stored at −20°C. Whole brains with pineals and pituitaries removed were placed in 3 ml of 70% isopropyl alcohol, emulsified with a hand-held homogenizer, centrifuged at 3000 × *g* for 10 min at 4°C, and the supernatant lyophilized and stored at −20°C. Processing controls were performed by the placement of I-PP in the bottom of tubes to which blood or brain from mice not injected with radioactive materials was introduced and processed as above. Samples were reconstituted in 0.1% trifluoroacetic acid (TFA) in water and analyzed by reversed-phase HPLC. Samples were eluted with a mixture of two phases that began as 70% of phase A (0.1% TFA in water) and 30% of phase B (0.1% TFA in acetonitrile) and increased to 50% of phase B over 60 min.

Octanol/Phosphate Buffer Partition Coefficients

A measure of the lipophilicity of I-PP was determined in a mixture of 2.5 (10^5) cpm of I-PP in 1 ml of octanol to which was added 1 ml of a 0.25 M phosphate buffer solution (PBS). This was mixed vigorously for 1 min, agitated for 10 min, and the two phases separated by centrifugation. Aliquots of 50 μl were taken in duplicate from each phase and counted. This experiment was done three times. The partition coefficient was expressed as the log of the ratio of cpm (octanol phase) to cpm (PBS phase).

Capillary Depletion Method

In other mice (n = 2/group), the entry of I-PP into the parenchymal/interstitial space of cerebral cortex or cerebellum depleted of capillaries was assessed as described for rats (46) and adapted to mice (31). Mice received IV injections of 10^6 cpm of I-PP and ^{99m}Tc-albumin (Medi + Physics albumin labeling kit, Paramus, NJ) with or without 100 μg/kg of unlabeled PP. Five minutes after IV injection, blood was collected from the carotid artery and the mouse was decapitated; the cerebral cortex and cerebellum were removed, weighed, and emulsified with a glass homogenizer (10 strokes) in 0.8 ml of physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, and 10 mM D-glucose adjusted to pH 7.4). Dextran solution (1.6 ml of a 26% solution) was added to the homogenate, which was vortexed and homogenized again (three strokes). Homogenization was performed at 4°C in less than 1 min. An aliquot of the homogenate was centrifuged at 5400 × *g* for 15 min at 4°C in a Beckman TL-100 ultracentrifuge with a TLS-55 swinging bucket rotor. The pellet or capillary fraction, containing brain vasculature, and the supernatant or parenchymal fraction were carefully separated and the levels of radioactivity determined in a gamma counter. The fractions were expressed as volumes of distribution in μl/g after correction for contamination by the vascular space as determined with ^{99m}Tc-albumin.

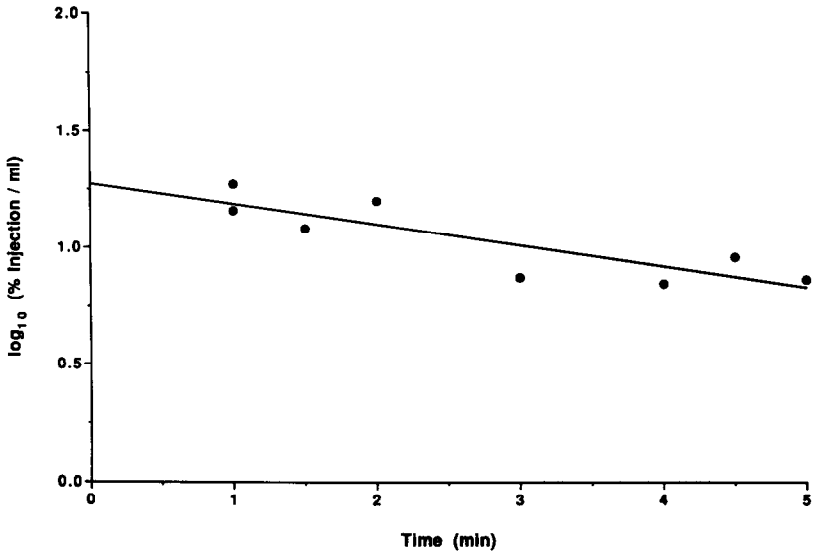


FIG. 1. Disappearance of I-PP from blood after IV injection. Results are expressed as log of the percent of injection in each ml of arterial serum vs. time. n = 8 mice.

Measurement of Brain to Blood Efflux Rates

Mice (n = 69) anesthetized with urethane were given injections of 1.0 μl I-PP into the left lateral ventricle of the brain as previously described (6). Mice were decapitated 1, 2, 5, 10, 15, 20, 25, or 30 min after the intracerebroventricular (ICV) injection and the level of radioactivity remaining in whole brain (minus pituitary and pineal) was measured in a gamma counter. The log(cpm/whole brain) was plotted against time and the half-time disappearance calculated from the slope. To test for inhibition, other mice (n = 83) had unlabeled PP at a dose of 10 or 50 ng/mouse or probenecid, tyrosine, sodium iodide, Tyr-MIF-1, somatostatin, arginine vasopressin (AVP), luteinizing hormone-releasing hormone (LHRH), or verapamil at a dose of 10 ng/mouse for each compound included in the ICV injection. These mice were decapitated at 10 min and compared with control mice not given candidate inhibitors decapitated at the same time.

Statistics

Regression lines were determined by the least-squares method and compared statistically by the use of the BMDP1R program (University of California Press, Berkeley, CA). Re-

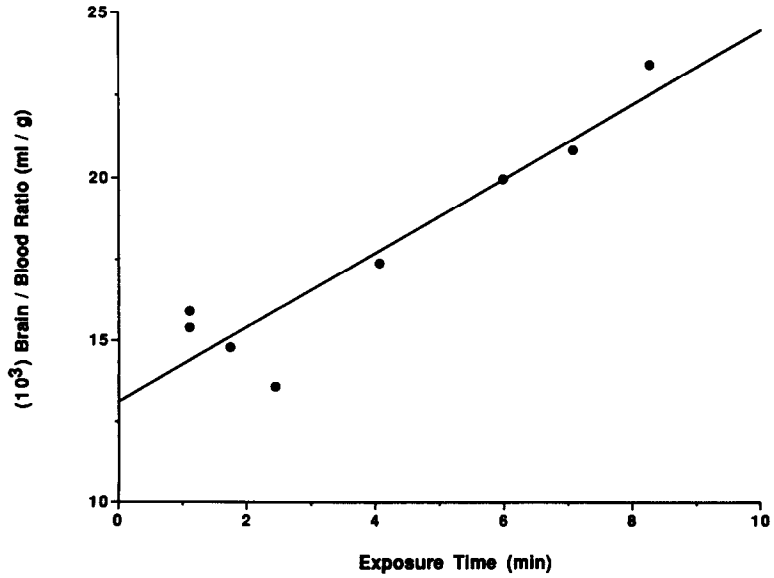


FIG. 2. Entry of I-PP into brain after IV injection. Results are expressed as the brain/blood ratio vs. exposure time by the method of multiple-time regression analysis. The slope measures K_i, the unidirectional influx constant, and the intercept measures V_i, the apparent volume of distribution in brain. n = 8 mice.

gression lines are reported with their correlation coefficients (r), the number of mice per line (n), and the level of statistical significance (p). Means are reported with their standard errors and ns and were compared by analysis of variance (ANOVA) followed by Duncan's multiple range test.

RESULTS

Figure 1 shows the relationship between levels of radioactivity in arterial serum vs. time after the IV injection of I-PP. This relationship had a slope of -0.0878 and an intercept of 1.27 ($n = 8$, $r = -0.847$, $p < 0.01$). This gave a half-time disappearance from the blood of 3.43 min and a volume of distribution of 5.38 ml, or 20–25% of body weight, indicating that I-PP is largely restricted to the vascular and extracellular spaces.

The relationship between brain/blood ratios and exposure time is shown in Fig. 2 for mice receiving an IV injection of I-PP. The K_i for this curve was 1.15 (10^{-3}) ml/g · min and the V_i was 13.1 μl/g ($r = 0.939$, $n = 8$, $p < 0.001$). Brain/blood ratios did not increase after 10-min exposure time, indicating that brain and blood had reached equilibrium, and are not shown.

Figure 3 shows the percent of the IV injection taken up by the brain during the first 5 min after IV injection. Levels peaked at 5 min at 0.065% injection/g of brain. Levels in the brain began to decline after 5 min and are not shown.

Addition of 3.5 μg/mouse of unlabeled PP to the IV injection reduced the K_i to 0.39 (10^{-3}) ml/g · min (Table 1). A statistically significant difference existed between the lines for mice receiving I-PP only and those receiving I-PP with unlabeled PP, $F(2, 11) = 12.7$, $p < 0.005$. Inclusion of unlabeled tyrosine in the IV injection had no effect on the entry of I-PP into the brain [$K_i = 1.32$ (10^{-3}) ml/g · min, $V_i = 10.9$ μl/g, $r = 0.905$, $n = 7$, $p = 0.005$]. Similar results were obtained in a separate experiment in which the group receiving 100 μg/mouse of unlabeled PP was also significantly different from the control group, $F(2, 15) = 9.21$, $p < 0.005$.

TABLE 1

EFFECT OF INCLUSION OF UNLABELED PANCREATIC POLYPEPTIDE (PP) OR THE AMINO ACID TYROSINE (Tyr) ON THE K_i AND V_i FOR INTRAVENOUSLY INJECTED [^{125}I]PANCREATIC POLYPEPTIDE (I-PP)

Material Coinjected With I-PP	$K_i(10^3)$	V_i	r	n
I-PP only	1.15	13.1	0.939	8
+ PP (3.5 μg)*	0.39	14.1	0.782	7
+ Tyr (3.5 μg)	1.32	10.9	0.905	7

K_i is given in ml/g · min and V_i in μl/g. r = regression coefficient, n = number of animals.

*Indicates a statistically significant difference ($p < 0.005$) from mice receiving I-PP only in their IV injection.

Table 2 shows the results for the uptake of I-PP by different brain regions. Overall, a statistically significant difference occurred among the groups, $F(8, 36) = 6.61$, $p < 0.00005$. The results for cerebellum were significantly different from those for pons-medulla ($p < 0.05$), cerebral cortex ($p < 0.005$), and whole brain ($p < 0.01$), and the results for pons-medulla were statistically different from those for cerebral cortex ($p < 0.005$) and whole brain ($p = 0.005$). The results for whole brain and cerebral cortex were not statistically different.

Figure 4 shows the chromatogram resulting from HPLC of brain tissue 10 min after IV injection of I-PP. Degradation controls were 75% intact for blood and 92% intact for brain.

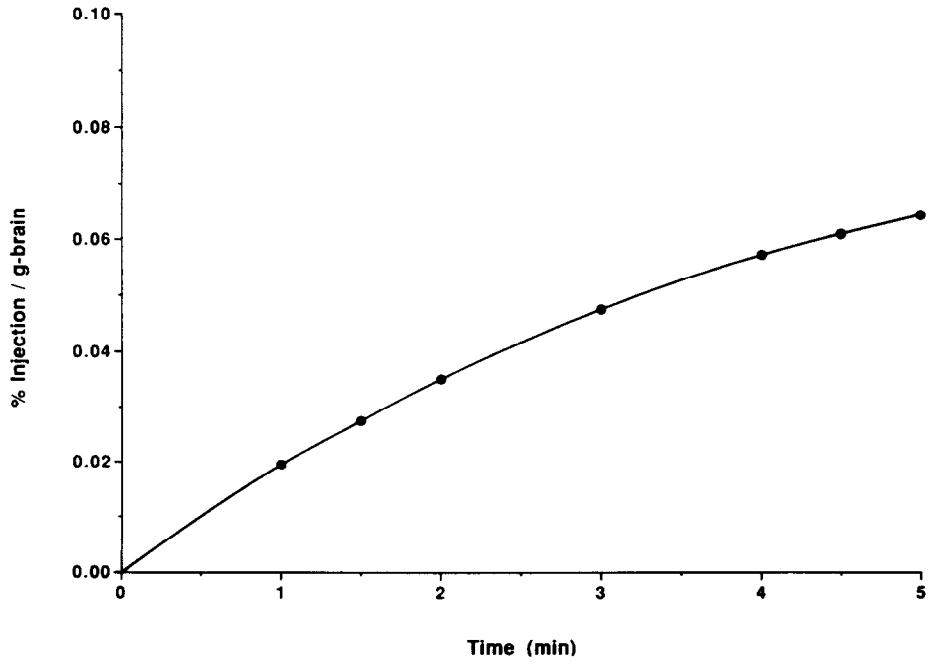


FIG. 3. Percent of the injection of I-PP found in each gram of brain vs. time after IV injection. n = 8 mice.

TABLE 2

K_i AND V_i FOR BRAIN REGIONS AFTER IV INJECTION OF I-PP

Brain Region	$K_i(10^3)$	V_i	r	n
Pons-medulla	2.06	0.42	0.824	8
Cerebellum	1.52	−0.37	0.940	10
Cerebral cortex	0.57	2.54	0.778	9
Remainder	0.64	2.20	0.782	9
Whole brain	0.64	2.81	0.830	10

Brains were washed free of vascular contents by means of cardiac perfusion.

K_i is given in ml/g · min and V_i in μl/g. r = regression coefficient, n = number of animals.

Correction with degradation controls gave 48% intact in brain 2 min after IV injection, 50% at 5 min, and 75% at 10 min, whereas in blood the values were 104% at 2 min, 84% at 5 min, and 32% at 20 min. The results for Fig. 2 and for the "I-PP only" column in Table 1 were recalculated after multiplication of the levels of radioactivity by percent intact. For those times when HPLC was not done, an extrapolation was used. For brain, no method exists to determine whether degradation products originated in the circulation, in which case levels of radioactivity in the brain should be corrected for the level of degradation, or whether they arose from intact peptide after passage across the BBB, in which case correction of levels of radioactivity in the brain for degradation would lead to underestimation of transport rate. When both brain and blood levels were corrected for degradation, the K_i decreased from 1.15 (10^{-3}) ml/g · min to 0.764 (10^{-3}) ml/g · min. When blood, but not brain, was corrected, the K_i increased to 1.50 (10^{-3}) ml/g · min.

The ratio of the radioactivity between the octanol and phosphate phases was 0.0477 ± 0.0072 (n = 3). This gave a partition coefficient with a log value of −1.32.

The results for the capillary depletion studies are shown in Fig. 5. A three-way ANOVA showed differences for compartment (i.e., parenchyma vs. capillaries), $F(1, 8) = 79.7$, $p < 0.001$, and for treatment (i.e., with or without unlabeled PP), $F(1, 8) = 42.3$, $p < 0.001$, but not for region (i.e., cortex vs. cerebellum). The range test showed that most of the I-PP was in the parenchymal fraction and that unlabeled PP significantly inhibited entry into this compartment.

After ICV injection of I-PP, a statistically significant relationship existed between log(cpm/whole brain) and time with a slope of −6.90 (10^{-3}) (n = 8, r = −0.929, $p < 0.001$) (Fig. 6). This gave a half-time disappearance from brain of 43.6 min. Efflux of I-PP from the brain was not affected by unlabeled PP or by any of the other substances tested.

DISCUSSION

The results support the hypothesis that modest amounts of blood-borne PP are able to cross the BBB to reach the parenchymal space of regions of the brain containing receptors for PP. Passage across the BBB occurs in part by means of a saturable transport system. This transport system has no brain to blood component, so that the efflux out of the CNS measured for PP is likely to be due to reabsorption of CSF.

The entry rate measured for I-PP is similar to that measured for other peptides (11). Entry of I-PP did not occur by nonsaturable membrane diffusion alone, but was due in large part to the presence of a saturable transport system. A dose of 3.5 μg of unlabeled PP was able to significantly inhibit transport; the saturable component of transport accounted for at least two-thirds of the rate of entry (Table 1). Based on the distribution volume of 5.38 ml measured for I-PP, we calculate that the initial level achieved in blood after the IV

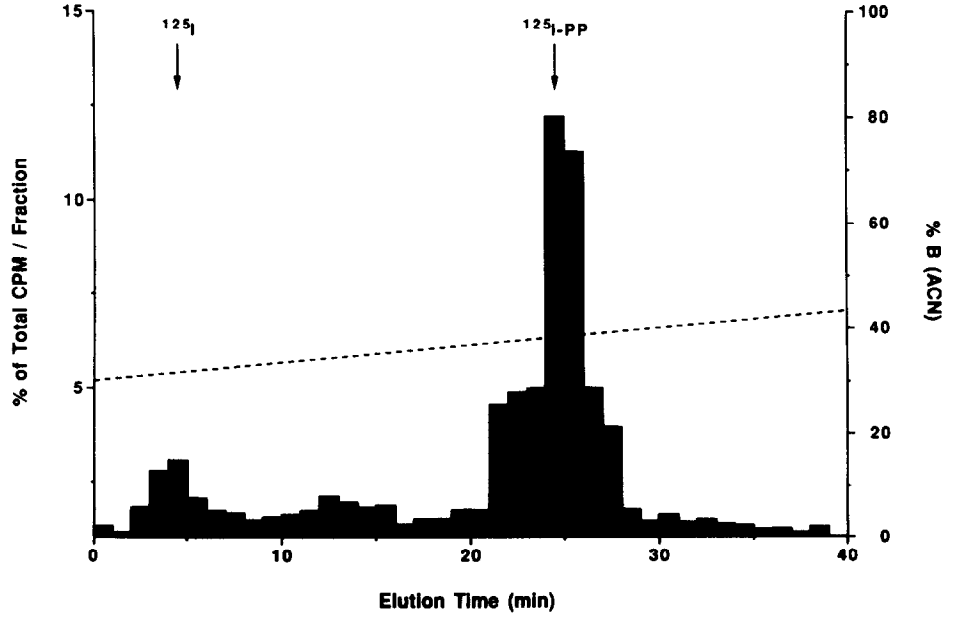
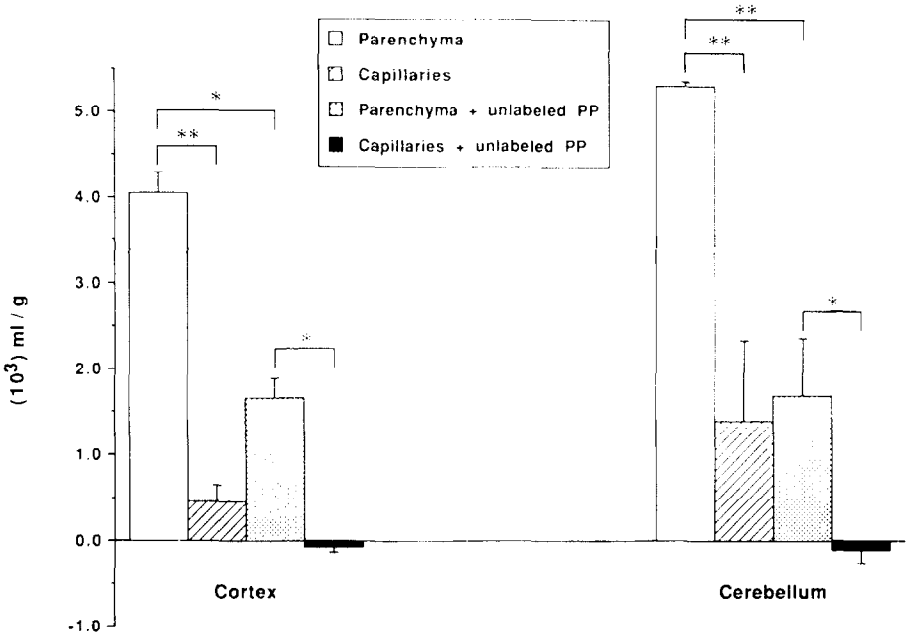


FIG. 4. Elution by HPLC of radioactivity recovered from brain 10 min after the IV injection of I-PP.



injection of 3.5 μg of unlabeled PP would be about 650 ng/ml. This greatly exceeds the peak serum concentrations of 0.362 ng/ml found in mice after cholinergic stimulation (32) and of 0.965 ng/ml found in dogs after insulin-induced hypoglycemia (34). The rate of nonsaturable transmembrane diffusion correlates with lipid solubility as measured by the octanol/PBS partition coefficient for many substances (45), including peptides (5). The nonsaturable transport rate indicated in Table 1 for I-PP is similar to that of β-amyloid (1-28), a peptide with a similar partition coefficient that is not transported across the BBB by a saturable system (12).

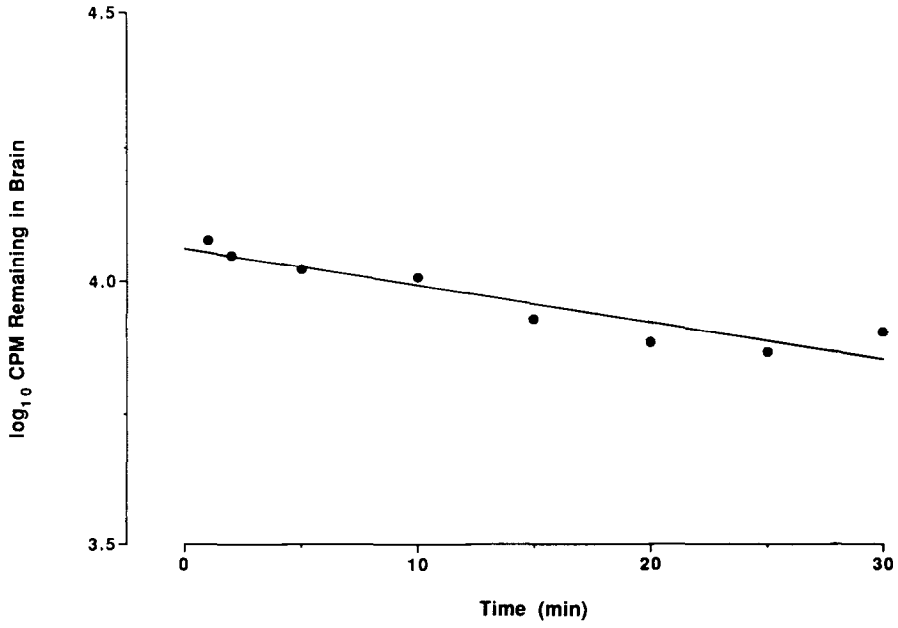


FIG. 6. Efflux of I-PP from brain after intracerebroventricular injection; each point represents a mean of eight to nine mice.

The results of the capillary depletion studies show that I-PP enters the parenchymal compartment of the brain rather than being sequestered by the endothelial cells of the capillary bed. The capillary depletion studies also confirm that entry into the parenchymal compartment is saturable. Some radioactivity was associated with the capillaries. This could represent binding of I-PP to endothelial receptors, but more likely represents I-PP in the process of being transported across the endothelial cells that comprise the BBB.

The ability of unlabeled PP, but not of the amino acid tyrosine, to inhibit transport of I-PP shows specificity to the inhibition. These doses are too low to interfere with the neutral amino acid transport of tyrosine, but the inability of tyrosine to inhibit I-PP at a dose that is effective for unlabeled PP argues that the amino acid transporter is not involved (41). HPLC confirmed that about 50% or more of the radioactivity recovered from the brain during the period of time studied represents intact peptide. This is consistent with the finding that most of the brain's enzymes that can degrade PP are in the cytosol. Therefore, the interstitial space of the brain is probably a relatively protected space for PP (1). The second largest peak on HPLC, which represented less than 10% of total radioactivity, eluted at the position of free iodide. Although it cannot be determined whether degradation products arose from the circulation or were produced within the brain, it is known that iodide is transported out of, but not into, the brain and that the brain contains a large amount of deiodinase. This favors the argument that the free iodide peak arose from I-PP that had crossed the BBB in intact form. Because deiodination could leave the PP molecule intact but not counted, our HPLC analysis of radioactive peaks may have overestimated the role of enzymatic degradation in preventing BBB penetration of PP.

The percent of the IV injection entering a gram of brain peaked 5 min after IV injection at slightly more than 0.06%. The percent entering the brain is influenced by several factors, including entry rate into the brain, total body volume of distribution, and half-time disappearance from blood. The value found here for I-PP is similar to that of other peptides that have ranged from 0.001 (48) to 1% (10) and exceeds that found for morphine by about threefold (4,10). Thus, although the amount of PP entering the brain is modest, it may be adequate to produce effects on brain function.

BBB permeability to I-PP differed among the various brain regions, as shown in Table 2. For this experiment, brains were perfused free of their vascular space before dissection. Such perfusion offers the advantage of correcting for regional differences in vascular space, negating any vasodilatory effects of the peptide, and removing the influence of uptake of the ligand by red blood cells. A disadvantage is that brain to blood passage may occur during this time. As a result of the perfusion, the V_is in Table 2 are much lower than those of Table 1. After perfusion, the residual V_is in cortex, for example, may represent incomplete perfusion or binding to endothelial cells. The low or slightly negative values for the V_is in pons-medulla and cerebellum suggest that almost all of the I-PP taken up by these tissues is sequestered in a compartment not in rapid equilibrium with blood.

The K_is in Table 2 show that the cerebellum and pons-medulla were significantly more permeable to I-PP than were the whole brain and cerebral cortex. By contrast, the peptides α-MSH and methionine enkephalin and the amino acid tyrosine cross the BBB less avidly at the cerebellum and pons-medulla than in some other regions of the brain (36,37). The cerebellum may be particularly sensitive to PP because of the large number of receptors there (2,3). Although parenchymal receptors are separate entities from endothelial transporters, the coordination of their locations may combine to target the efficacy of blood-borne I-PP.

These results are consistent with previous work by others (51) showing that I-PP can reach the CVOs. The lack of a BBB within the CVOs allows rapid entry of circulating radioactivity, but circumscription of the CVOs by the BBB prevents diffusion of the accumulated radioactivity. As a result, the CVOs are easily detected by autoradiography. However, autoradiography, as generally used, is much less sensitive than the methods of detection applied here, which are so sensitive that they can even quantify the entry of substances commonly used as vascular markers (25,43). When the total amount of radioactivity entering the brain is considered, it becomes obvious that the areas containing CVOs often account for much less uptake of labeled compound than the less permeable, but larger, areas behind the BBB that are free of CVOs, such as the cerebellum and cortex (14). It is probable, therefore, that blood-borne PP is able to reach receptors at both the CVOs and in areas of the CNS behind the BBB.

The efflux of I-PP from the brain is nonsaturable and consequently probably occurs by reabsorption of the CSF (42). The half-time disappearance from brain of 43.6 min is about the same as that of albumin (15), which is also reabsorbed by this route (26). This slow rate of exit is consistent with the previous finding of a slow decline in levels of PP in the CSF once they were elevated (34). However, this nonspecific efflux system can still account for significant appearance of centrally administered materials in the blood. Efflux also was not inhibited by probenecid, tyrosine, sodium iodide, Tyr-MIF-1, somatostatin, AVP, LHRH, or verapamil, indicating that efflux did not occur through the transport systems described for organic acids (21), neutral amino acids (41), anions (52), Tyr-MIF-1/methionine enkephalin (16,18), somatostatin (20), AVP (17), LHRH (22), or the *P*-glycoprotein multidrug resistance pump (47) respectively.

Although bovine PP was used as the radioactive ligand, uptake was studied in the mouse. It is possible that murine PP may differ enough from bovine PP that the uptake might also differ. By example, the rate of transport across the mouse BBB of murine interleukin-1α is about four times greater than that of human interleukin-1α (19). The catabolism of murine PP by blood and peripheral organs might also be different from that of bovine PP. Such peripheral factors, by altering the amount of peptide presented to the brain, can have important effects in determination of the amount of material entering the brain (8).

In conclusion, I-PP is transported into the brain by a saturable transporter that shows regional variation. Transport is particularly rapid into the cerebellum, an area rich in receptors for PP, and the pons-medulla. The peptide is transported intact into the parenchymal compartment of the brain. Efflux of I-PP out of the brain is by a nonsaturable process and probably occurs with reabsorption of the CSF. The transport system described could explain how blood-borne PP can affect the CNS.

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