Pharmacologic effects *in vivo* in brain by vector-mediated peptide drug delivery

(vasoactive intestinal peptide/blood–brain barrier/cerebral blood flow)

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ABSTRACT   Pharmacologic effects in brain caused by systemic administration of neuropeptides are prevented by poor transport of the peptide through the brain vascular endothelium, which comprises the blood–brain barrier *in vivo*. In the present study, successful application of a chimeric peptide approach to enhance drug delivery through the blood–brain barrier for the purpose of achieving a central nervous system pharmacologic effect is described. The chimeric peptide was formed by linkage of a potent vasoactive intestinal peptide (VIP) analogue, which had been monobiotinylated, to a drug transport vector. The vector consisted of a covalent conjugate of avidin and the OX26 monoclonal antibody to the transferrin receptor. Owing to the high concentration of transferrin receptors on brain capillary endothelia, OX26 targets brain and undergoes receptor-mediated transcytosis through the blood–brain barrier. Systemic infusion of low doses (12 μg/kg) of the VIP chimeric peptide in rats resulted in an *in vivo* central nervous system pharmacologic effect: a 65% increase in cerebral blood flow. Biotinylated VIP analogue without the brain transport vector was ineffective.

Morphological, biochemical, and functional evidence indicates that vasoactive intestinal polypeptide (VIP) is a potent cerebrovascular vasodilator (1–6). Although VIP causes vasodilation *in vitro* (7, 8) and when applied locally to intracranial blood vessels (9), no consistent cerebrovascular effects could be demonstrated after systemic application of the peptide (10–12). This exemplifies the prohibitive role played by the blood–brain barrier (BBB) in limiting peptide transport to brain and subsequent neuropharmacologic effects. The use of chimeric peptides offers an approach to enhanced brain delivery of such peptides: drugs that normally do not cross the BBB may be coupled to brain drug transport vectors (13).

Brain drug transport vectors include proteins such as cationized albumin (14) or the OX26 monoclonal antibody (15), which undergo absorptive- or receptor-mediated transcytosis through the BBB, respectively. OX26 is a murine monoclonal antibody directed against the rat transferrin receptor (16). The transferrin receptor is highly expressed on brain capillary endothelium (16, 17) and allows for receptor-mediated transcytosis of circulating transferrin (18) or the OX26 monoclonal antibody (15, 19) through the BBB.

Coupling of drugs to brain transport vectors such as OX26 is facilitated by use of the avidin–biotin system (20), and coupling of avidin to the transport vectors enables their potential application to delivery of biotinylated peptides or antisense oligonucleotides (21, 22). However, an essential requirement for a biotinylated ligand of an avidin transport vector conjugate is monobiotinylation. Higher degrees of

biotinylation inevitably cause aggregation by avidin, which has four binding sites for biotin (23). Therefore, in the present studies, a VIP analogue (VIPa) suitable for monobiotinylation was developed. To demonstrate an *in vivo* pharmacologic effect with the VIPa, cerebral blood flow in rats was measured after short-term systemic infusion of the VIPa, either alone or coupled to the BBB transport vector.

MATERIALS AND METHODS

Materials. Mammalian VIP was purchased from Peninsula Laboratories. Na^{125}I (specific activity, 2050 Ci/mmol; 1 Ci = 37 GBq) and (3-[^{125}I]iodotyrosyl^{10})VIP (specific activity, 2000 Ci/mmol), referred to here as ^{125}I-VIP, were obtained from Amersham. [^{3}H]Borohydride (specific activity, 9.8 Ci/mmol) was obtained from DuPont/NEN. Soluene-350 was obtained from Packard. Sulfosuccinimidyl 2-(biotinamido)ethyl-1,3'-dithiopropionate (NHS-SS-biotin), 2-iminothiolane (Traut's reagent), *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, trifluoroacetic acid, and BCA protein assay reagents were purchased from Pierce. Protein G-Sepharose 4 Fast Flow was supplied by Pharmacia LKB. Triethylamine was obtained from Aldrich and endoproteinase Lys-C was from Boehringer Mannheim. HPLC columns were products of The Separations Group (Vydac C_4) or of PolyLC (Columbia, MD) (polyhydroxyethyl A). Male Sprague–Dawley rats (200–300 g) were obtained from Harlan–Sprague Dawley. Avidin and other reagents were from Sigma.

Preparation of VIPa. [*N*-acetyl-His^1,Nle^{17},Arg^{20},Arg^{21}, Ala^{26}]VIP (VIPa; Fig. 1A) was prepared by the UCLA Peptide Synthesis Facility. The crude peptide was purified on a preparative reverse-phase HPLC column (Vydac C_4; 2.2 × 25 cm) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Amino acid analysis confirmed the desired amino acid composition.

Biotinylation and Desbiotinylation of VIPa. VIPa (500 μg; 150 nmol) was dissolved in 1 ml of 0.125 M NaHCO$_3$ in distilled H$_2$O (pH 8.3) and mixed with a 10-fold molar excess of the cleavable biotin linker NHS-SS-biotin (910 μg in 1 ml of 0.125 M NaHCO$_3$). The reaction was allowed to proceed for 1 h at room temperature and was then stopped by addition of 1 ml of 5% trifluoroacetic acid. Separation of the reaction products was performed on a reverse-phase HPLC column (Vydac C_4; 1 × 25 cm) at a flow rate of 3 ml/min and a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Absor-

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Abbreviations: VIP, vasoactive intestinal peptide; VIPa, VIP analogue; bioVIPa, biotinylated VIPa; desbioVIPa, desbiotinylated VIPa; BBB, blood–brain barrier; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3'-dithiopropionate; FAB, fast atom bombardment; V$_D$, volume of distribution.
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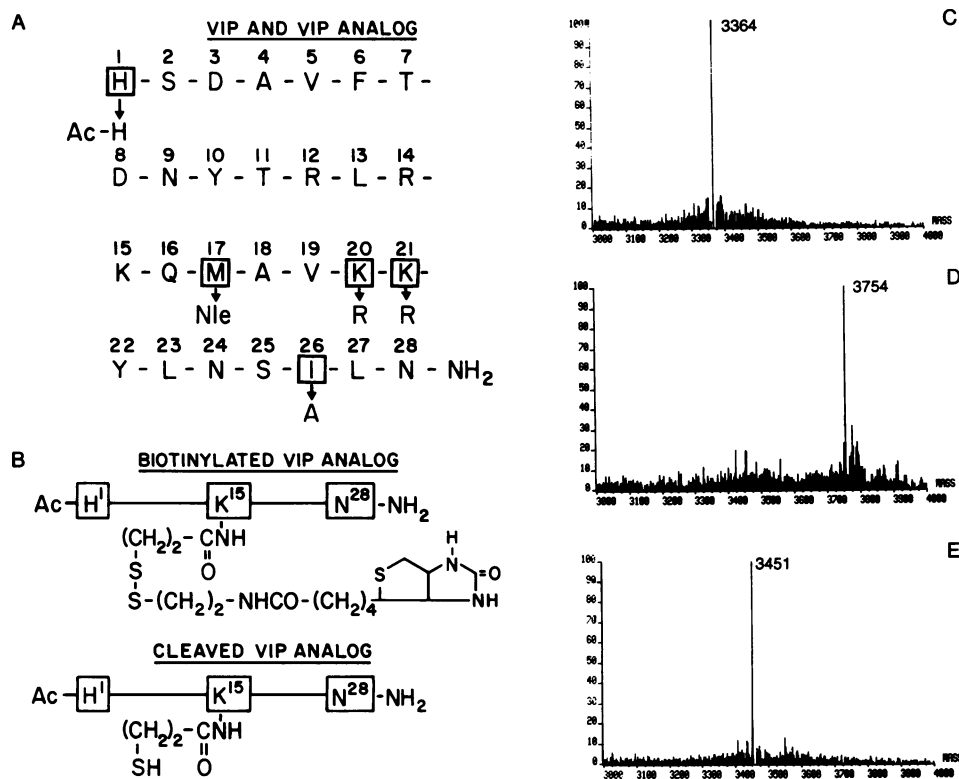
FIG. 1. (A) Amino acid sequence of native VIP and modifications in VIPa as indicated by arrows. (B) Structure of biotinylated VIPa (bioVIPa) and desbiotinylated VIPa (desbioVIPa). (C–E) Molecular mass as determined by fast atom bombardment (FAB) MS of HPLC purified VIPa (C), bioVIPa (D), and desbioVIPa (E).

bance was monitored at λ = 214 nm. The structure of biotinylated VIPa (bioVIPa) is shown in Fig. 1B.

For cleavage of the biotin moiety from bioVIPa, 44 μg of this peptide was dissolved in 200 μl of 0.01 M sodium phosphate buffer (pH 7.4) containing 50 mM dithiothreitol as a disulfide reducing agent. After incubation for 1 h at room temperature, 800 μl of 0.5% trifluoroacetic acid was added. The reaction mixture was separated on the HPLC system described above. The desbiotinylated peptide derivative (desbioVIPa) has a mercaptopropionate residue attached to the ε-amino group of Lys[15], as shown in Fig. 1B.

Fast atom bombardment (FAB) mass spectra of the HPLC purified fractions were obtained by using xenon (8 kV; 100 μA) as the bombarding gas with the instrument (VG ZAB-SE) set at 8 kV accelerating potential and with a mass resolution of 500.

VIP Radioreceptor Assay. The biological activity of VIPa, bioVIPa, and desbioVIPa was assessed with a rat lung VIP radioreceptor assay. Rat lung membranes were prepared as described by Leroux *et al.* (24). Binding experiments were performed at 37°C for 20 min with 125 μg of rat lung membrane protein in a total vol of 500 μl. The assay buffer consisted of 25 mM Tris·HCl, pH 7.4/5 mM MgCl$_2$/0.2% bovine serum albumin/0.1% bacitracin/^{125}I-VIP ($\approx$10,000 cpm; final concentrations, 3.9–6.6 pM). The bound fraction was separated by centrifugation for 5 min at 10,000 × g at 4°C. Binding was expressed as the ratio of bound to total counts at each concentration of nonlabeled peptide. All displacement curves were fit simultaneously by weighted least squares using the nonlinear regression program BMDP3R (25). Predicted bound fractions were computed by a Newton–Raphson technique for a two-site model (specific high- and low-affinity sites, one nonspecific site) with one labeled and one unlabeled ligand (26). Binding capacities and percentage nonspecific binding were assumed independent of ligand species but were rescaled in each experiment to account for

minor differences in receptor concentration. After examining standardized residuals under several error variance models, weights inversely proportional to the bound fraction were chosen. The data were also fit to a one-site model; however, a formal F test rejects this in favor of a two-site model ($P <$ 0.0001).

Iodination of VIPa and Biotinylation. The transport of ^{125}I-bioVIPa through the BBB attached either to avidin or to the avidin–OX26 conjugate was studied with an internal carotid artery infusion technique (see below). VIPa was first iodinated with Na[125]I and Na[127]I (molar ratio of ^{125}I/[127]I, 1:60) using a mild chloramine-T procedure (molar ratios of peptide/iodine/chloramine T, 1:1:5). The HPLC-purified, iodinated VIPa was further characterized by enzymatic digestion with endoproteinase Lys-C, which cleaves VIPa at the single Lys[15] residue, yielding peptide fragments 1–15 and 16–28. HPLC analysis of the iodinated VIPa revealed a total of four peaks, two of which coeluted with fragments of native VIPa. The two additional fragments most likely correspond to [Tyr(I)[10]]VIPa 1–15 and [Tyr(I)[22]]VIPa 16–28, indicating that the iodinated VIPa peak actually consists of [Tyr(I)[10]]VIPa 1–28 and [Tyr(I)[22]]VIPa 1–28. It has been demonstrated for native VIP that both iodinated species [Tyr(I)[10]]VIP 1–28 and [Tyr(I)[22]]VIP 1–28 are bioactive (27).

NHS-SS-biotin (150 nmol) in 100 μl of 0.125 M NaHCO$_3$ (pH 8.3) was added to 7.5 nmol of lyophilized ^{125}I-VIPa. After incubation for 2 h at room temperature, 1 μl of concentrated phosphoric acid and 100 μl of acetonitrile were added. ^{125}I-bioVIPa was purified by hydrophilic interaction chromatography on a polyhydroxyethyl A column (0.46 × 20 cm). The elution system consisted of 50 mM triethylamine phosphate (pH 2.8) and 60% acetonitrile/50 mM triethylamine phosphate, pH 2.8. A linear decreasing gradient of acetonitrile from 60% to 30% over 20 min was run at a flow rate of 1 ml/min.
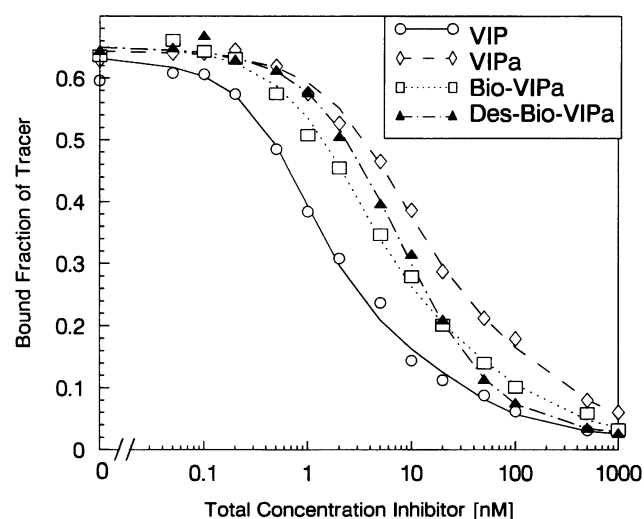
FIG. 2. Competition curves in the radioreceptor assay using a preparation of rat lung membrane, ^{125}I-VIP tracer, and nonradioactive inhibitors in concentrations given on the x axis. Experimental points represent means of duplicate determinations. Coefficient of variation was <5% over the entire range of concentrations.

Synthesis of Avidin–OX26 Conjugate. OX26 was purified from ascitic fluid produced in BALB/c mice with protein G-Sepharose 4 Fast Flow (20). Conjugation of OX26 and avidin was accomplished with 2-iminothiolane for thiolation of avidin and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester activation of OX26 as described (20). The conjugate was purified on a Superose 6 HR gel-filtration column. The molar ratio of avidin/OX26 was ≈1:1.

BBB Transcytosis *in Vivo*. BBB transport of ^{125}I-bioVIPa coupled either to avidin or to an avidin–OX26 conjugate was measured with an internal carotid artery perfusion/capillary depletion technique as described (28). The perfusate solutions were prepared in Krebs–Henseleit buffer (pH 7.4; 118 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/25 mM $NaHCO_3$/10 mM D-glucose/0.1 g of bovine serum albumin per dl). Avidin–OX26 conjugate or avidin was added to a final concentration of 28 nM together with ^{125}I-bioVIPa (final concentration, 13.1 nM; 0.4 μCi/ml). The perfusate also contained [3H]-labeled rat serum albumin as a blood volume marker at 5 μCi/ml. [3H]-labeled rat serum albumin (specific activity, 0.11 Ci/mol) was prepared as described (29). Rats were anesthetized with ketamine and maintained at 37°C with a thermal blanket (Harvard).

Effects of bioVIPa on Tissue Blood Flow in Rats. As modulation of cerebral blood flow may be impaired under ketamine anesthesia, rats were tracheotomized and artificially ventilated with nitrous oxide/oxygen throughout the blood flow measurement. Under halothane anesthesia, the right external carotid artery was retrogradely cannulated and the right pterygopalatine, superior thyroid, and occipital arteries were occluded. The left femoral artery was also cannulated.

Table 1. VIP receptor parameters

Ligand	B_{max}, pmol·mg⁻¹	K_d, nM	K_i, nM
VIP	1.6 ± 0.2	0.26 ± 0.04	—
VIPa	—	—	3.2 ± 0.3
BioVIPa	—	—	1.0 ± 0.2
DesbioVIPa	—	—	1.6 ± 0.2

Nonspecific binding = 2.2% ± 0.2%. Parameters were estimated by fitting data in Fig. 2 to a two-site binding model by nonlinear regression analysis. Low-affinity B_{max} = 16 ± 3 pmol·mg⁻¹; low-affinity VIP K_d = 23 ± 6 nM; low-affinity K_i = 207 ± 42, 66 ± 17, and 16 ± 4 nM, for VIPa, bioVIPa, and desbioVIPa, respectively. Estimates are ±1 SE.

Atropine sulfate (0.1 mg/kg) and tubocurarine chloride (0.5 mg/kg) were injected i.v. The animals were then tracheotomized and artificially ventilated with 70% nitrous oxide/30% oxygen; body temperature was maintained at 37°C with a thermal blanket. After 30 min, arterial blood gas analysis was performed, and if the results showed normocapnia (P_{CO_2} between 30 and 40 mmHg), intracarotid infusions were started. The four treatment groups received vehicle (Krebs–Henseleit buffer) containing 1% bovine serum albumin, bio-VIPa (3 μg/ml), avidin–OX26 (110 μg/ml), or bioVIPa–avidin–OX26 conjugate (3 μg of bioVIPa and 110 μg of avidin–OX26 per ml) at a constant infusion rate (0.11 ml/min) over 10 min. At the end of the infusion, [3H]diazepam and [14C]sucrose in saline were injected i.v. into the right femoral vein; blood was collected from the femoral artery for 10 sec, after which the animals were decapitated. Thyroid or brain hemisphere blood flow was calculated as the ratio of [3H]diazepam radioactivity in tissue versus [3H]diazepam integrated radioactivity in the arterial blood sample.

RESULTS

Biotinylation of VIPa with NHS-SS-biotin yielded an efficiency of 80% under the chosen reaction conditions. This was calculated from the ratio of the peak areas (bioVIPa/VIPa) in
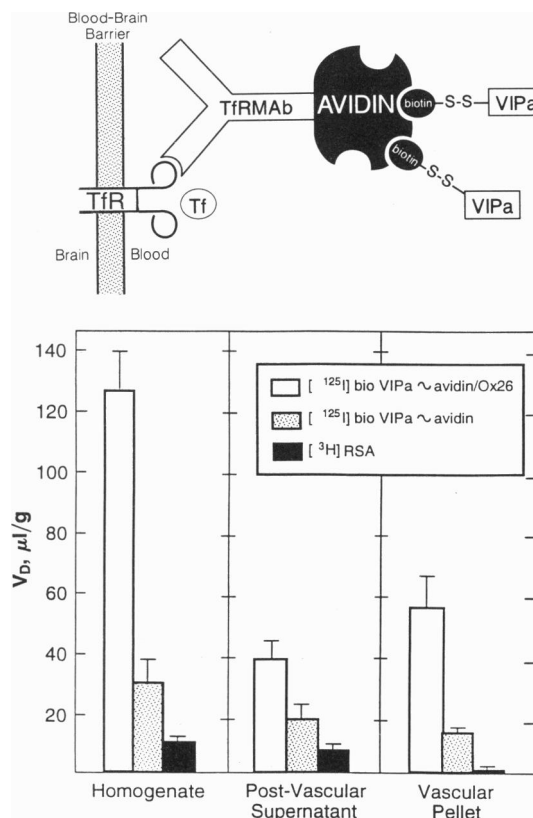

FIG. 3. (*Upper*) Delivery of bioVIPa (see Fig. 1*B*) through the BBB using a transport vector composed of a conjugate between avidin and the OX26 antitransferrin receptor monoclonal antibody (TfRMAb). Tf, circulating transferrin; TfR, BBB transferrin receptor. (*Lower*) Brain uptake in rats of tracers (see *Inset*) as determined by an internal carotid artery perfusion/capillary depletion technique. Tissue uptake is expressed as volume of distribution in μl/g at the end of a 10-min perfusion with tracer solution containing either ^{125}I-bioVIPa–avidin–OX26 and [3H]-labeled rat serum albumin ([3H]RSA) or ^{125}I-bioVIPa–avidin and [3H]RSA (*n* = 3 per group). Results are shown for whole tissue homogenate, postvascular supernatant of homogenate, and vascular pellet. Results of the two groups for [3H]RSA were not significantly different and were combined for the evaluation. Bars represent means ± SE.

the HPLC chromatogram. Chemical cleavage of the disulfide bond with the reducing agent dithiothreitol completely converted bioVIPa into desbioVIPa, as no bioVIPa was detectable with HPLC. FAB MS (Fig. 1 *C–E*) of the HPLC-purified peptides VIPa, bioVIPa, and desbioVIPa resulted in the following mass/charge ratios (m/z) for the single charged protonated molecules (MH^+): 3364, VIPa; 3754, bioVIPa; and 3451, desbioVIPa. The corresponding mass values calculated from the chemical formulas are 3362.7, 3752.3, and 3450.8 Da, respectively.

The displacement curves obtained in the radioligand binding assay are shown in Fig. 2. Nonlinear regression analysis of the data showed that the inhibitor constants of the VIPa analogue and its biotinylated and desbiotinylated derivatives were ≈1 order of magnitude higher compared to native VIP (Table 1).

The tracer for the brain uptake experiments was purified by hydrophilic interaction chromatography on a polyhydroxyethyl A column, as it proved difficult to recover ^{125}I-bioVIPa from reverse-phase HPLC columns. This type of chromatography has recently been described to be especially suitable for separation of hydrophilic or basic peptides, such as VIP (30). The results of the internal carotid artery perfusion method are shown in Fig. 3. As preliminary studies with ^{125}I-VIP analogues had shown no measurable transcytosis through the BBB, subsequent experiments evaluated the transport of ^{125}I-bioVIPa coupled either to avidin or to the avidin–OX26 conjugate. Both the coupling to avidin and to avidin–OX26 vector conjugate resulted in significant brain uptake of the peptide compared to the vascular marker ^3H-labeled rat serum albumin (Fig. 3); 91% of the tracer in the postvascular supernatant was precipitable with trichloroacetic acid, indicating that the label was still attached to macromolecules and did not represent metabolic breakdown products such as iodotyrosine or free iodine. After correction for trichloroacetic acid precipitability and intravascular content, the volume of distribution (V_D) of ^{125}I-bioVIPa–avidin–OX26 in brain postvascular supernatant was 32 ± 7 $\mu l/g$ compared to 12 ± 6 $\mu l/g$ for ^{125}I-bioVIPa–avidin. Converted to fraction of injected dose (ID) entering the brain tissue beyond the BBB, these V_D values equal an accumulation of 0.256% ID/g and 0.096% ID/g, respectively. Approximately the same amounts of peptide were associated with the vascular compartment.

The pharmacologic effects of the VIPa on tissue blood flow are shown in Fig. 4. Pilot studies with i.v. bolus injection of [^3H]diazepam and [^{14}C]sucrose had shown that the tracer completes the first capillary passage through the circulation within 10 sec. Thus, a 10-sec sampling time was adopted for the cerebral blood flow measurements (Fig. 4*A*). All experimental animals in the different treatment groups showed normocapnia and physiological pH values. The pooled data are represented in Fig. 4*B*. Cerebral blood flow in the control group (infusion of Krebs–Henseleit buffer only) was 2.18 ± 0.04 ml·min^{-1}·g^{-1} (Fig. 4*D*). No effects on cerebral blood flow could be observed after separate infusions of either bioVIPa (12 $\mu g/kg$) or the transport vector avidin–OX26 (430 $\mu g/kg$). BioVIPa, however, significantly increased the blood flow rate through the thyroid gland from 3.50 ± 0.26 ml·min^{-1}·g^{-1} to 6.62 ± 1.47 ml·min^{-1}·g^{-1} (Fig. 4*C*). When bioVIPa was coupled to avidin–OX26, vector-mediated brain uptake of the peptide resulted in a significant 65% increase in brain blood flow to 3.59 ± 0.27 ml·min^{-1}·g^{-1} (Fig. 4*D*). The volume of distribution of the intravascular marker [^{14}C]sucrose in brain tissue showed no difference in all groups and averaged 6.2 ± 0.7 $\mu l/g$. This indicates that the permeability of the BBB was not changed and that cerebral blood flow was not increased through capillary recruitment, which would increase the sucrose V_D in brain.

DISCUSSION

The present studies describe the design of a VIPa suitable for delivery through the BBB with an avidin–vector conjugate. The primary goals in this design were (*i*) selective monobiotinylation with the cleavable biotin linker NHS-SS-biotin, and (*ii*) biotinylation at a site on the peptide that is not critical to VIP receptor binding. Although the free N-terminal α-amino group of native VIP is not easily conjugated by biotinylation reagents (31), it was still blocked by acetylation. This modification also renders the peptide less susceptible to aminopeptidase attack, thus contributing to enhanced metabolic stability. Moreover, N^α-acetylation does not adversely affect the bioactivity of VIP (32). The lysine residues at positions 20 and 21 of native VIP were substituted by arginine, similar to basic amino acid substitutions made in dynorphin analogues (33). These changes left the ε-amino group of lysine at position 15 as the only possible site for biotinylation. It has been demonstrated that biotinylation of Lys^{15} does not markedly decrease receptor binding properties or biological activities of VIP (31). The substitution of Ala^{26} for Ile^{26} has recently been found to prolong the duration
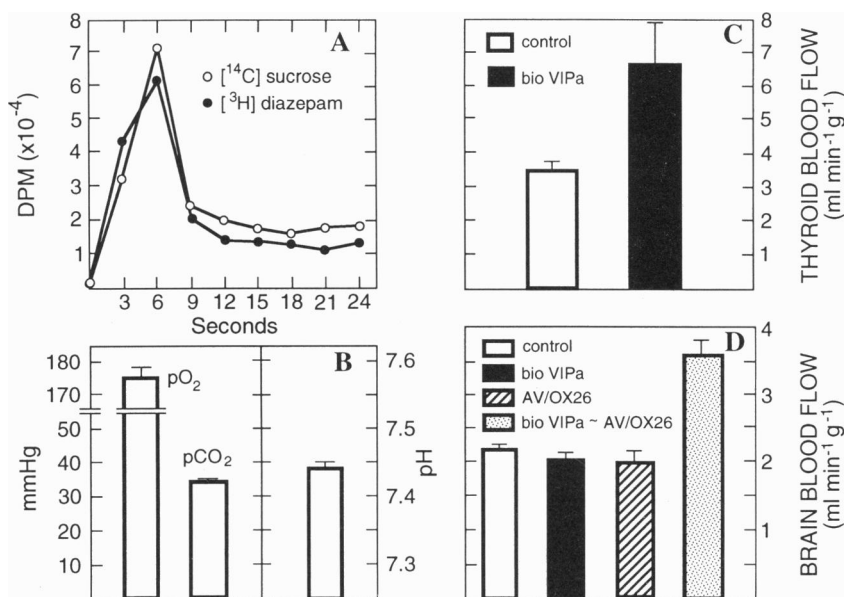

FIG. 4. Measurement of tissue blood flow after 10-min intracarotid infusions of vehicle (control), bioVIPa (12 $\mu g/kg$), avidin–OX26 (430 $\mu g/kg$), or bioVIPa–avidin–OX26 (12 μg of bioVIPa and 430 μg of avidin–OX26 per kg) in N_2O anesthetized rats. (*A*) Arterial blood radioactivity of [^3H]diazepam or [^{14}C]sucrose was determined in sampling periods of 3 sec after i.v. bolus injection. (*B*) No significant differences in arterial blood gas measurements of the four treatment groups listed in *D* were observed and means ± SE for all animals (n = 16) are shown. (*C*) Rate of thyroid blood flow is increased by systemic administration of bioVIPa over 10-min infusion period. (*D*) Brain blood flow is increased 65% (P < 0.0025; Student's t test versus control) by systemic administration of bioVIPa coupled to the avidin–OX26 (AV/OX26) vector, whereas administration of either bioVIPa or AV/OX26 vector alone causes no change in brain blood flow relative to control. No difference in blood flow between right and left hemispheres was found. Data are means ± SE (n = 4 rats per group; body weight, 270–280 g).

of action of VIP considerably, without appreciable loss of bioactivity (34). The introduction of norleucine instead of methionine at position 17 is advantageous in peptides subjected to the oxidative reaction conditions of iodination.

The resulting synthetic VIPa could efficiently be biotinylated with NHS-SS-biotin. The disulfide bond present in this linker allows cleavage of the biotin moiety from the peptide, which could be demonstrated by treatment with dithiothreitol (Fig. 1). Previous studies have shown that the disulfide linkage is rapidly cleaved in brain but not in brain capillaries (13). Feener *et al.* (35) have demonstrated conjugate disulfide cleavage activity in the Golgi apparatus but not in endosomes or lysosomes.

The internal carotid artery perfusion study with ^{125}I-bioVIPa showed a tissue accumulation of 3.2% of the perfusate concentration at the end of the 10-min perfusion period when the peptide was coupled to the avidin–OX26 transport vector. The modest uptake of the peptide conjugated to avidin alone (Fig. 3) is explained by the cationic nature of avidin, which leads to an absorptive-mediated endocytosis by brain endothelial cells (21). However, avidin itself has a limited usefulness as a brain drug transport vector due to its rapid systemic clearance by liver and kidney (22), resulting in a low plasma integral following systemic administration compared to avidin–OX26 (36). Moreover, a comparison of the permeability–surface area products showed that the rate of brain uptake of [^{3}H]biotin–avidin is only 22% of [^{3}H]biotin–avidin–OX26 (36). Assuming an internal carotid artery plasma flow on the order of the perfusion rate in the tracer experiments of 1.25 ml/min, the peptide solution in the cerebral blood flow experiments (3 μg of bioVIPa per ml = 0.8 μM at an infusion rate of 0.11 ml/min) would have been diluted ≈1:11 to a plasma concentration of ≈70 nM. If the brain uptake in this experiment was comparable to the tracer uptake, then the brain tissue concentration in the postvascular compartment, which caused the 65% increase in cerebral blood flow (Fig. 4D), was ≈2.25 pmol per g of brain at the end of the 10-min infusion. While there was no effect on cerebral blood flow elicited by the same dose of bioVIPa without transport vector, the systemic concentrations of the peptide were sufficient for an almost 2-fold increase in thyroid blood flow (Fig. 4C). This result provides evidence for the bioactivity of the VIPa used in the present studies and is consistent with previous reports demonstrating the effect of VIP and structurally related hormones on blood flow in peripheral glands lacking a vascular barrier, such as pancreas, salivary glands, and thyroid (37). On the other hand, intracarotid bioVIPa has no effect on brain blood flow because cerebrovascular VIP receptors are located on vascular smooth muscle cells beyond the BBB.

In conclusion, the present study demonstrates the successful application of the drug transport vector avidin–OX26 for brain delivery of a monobiotinylated VIPa. When this delivery system, which may also transport other monobiotinylated drugs, was used, systemic administration of a small dose of the neuropeptide resulted in an *in vivo* central nervous system pharmacologic effect—increase of cerebral blood flow.

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