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In Vivo Cleavability of a Disulfide-Based Chimeric Opioid Peptide in Rat Brain

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Brain delivery of systemically administered neuropeptide drugs may be achieved by the synthesis of chimeric peptides, wherein the peptide is coupled to transport vectors via avidin–biotin technology. The present study focuses on factors that optimize the linkage of drugs to transport vectors. The vector is the OX26 monoclonal antibody to the transferrin receptor, and the model peptide used in these studies is [Lys^7]dermorphin (K7DA). The K7DA is monobiotinylated at the ϵ-amino group of the Lys^7 residue with either a cleavable linker, e.g., disulfide, using NHS-SS-biotin, or a noncleavable linker, e.g., amide, using NHS-XX-biotin. Disulfide cleavage of the biotinylated derivative yields the desbiotinylated peptide, which is thiolated. Structures of the K7DA analogues were confirmed by secondary ion mass spectrometry. The biotinylated peptides were coupled to a thiol–ether conjugate of the OX26 antibody and either neutral avidin (NLA) or streptavidin. The binding constants (K_i) of the K7DA, the biotinylated K7DA (bio-XX-K7DA), the desbiotinylated K7DA, and the bio-XX- KD7A conjugated to NLA-OX26 were 0.62 ± 0.14, 1.59 ± 0.27, 1.24 ± 0.24, and >10 nM, respectively, and were determined with a μ-opioid peptide radioreceptor assay. Comparable results were obtained with in vivo tail-flick analgesia testing following intracerebroventricular (icv) injection of opioid chimeric peptides. Reversibility of pharmacologic action of thiolated peptide was demonstrated by icv naloxone administration. The cleavability of the disulfide linker in vivo in rat plasma and brain was assessed with gel filtration HPLC and internal carotid artery perfusion of labeled opioid chimeric peptides. These studies are consistent with the following conclusions: (a) opioid peptides have minimal pharmacologic activity when bound to the transport vector, indicating the need for cleavable disulfide linker; (b) the disulfide linker is stable in plasma in vivo as well as brain capillary endothelial cells, but is rapidly cleaved in rat brain in vivo, indicating that disulfide cleavage occurs beyond the endothelial cells of brain capillaries; and (c) the thiolated peptide released following disulfide cleavage is pharmacologically active at the μ-opioid peptide receptor via a naloxone reversible mechanism, indicating the thiolated peptide is not likely covalently bound to the receptor.

INTRODUCTION

The brain capillary endothelial wall, which is the anatomical basis of the blood–brain barrier (BBB) in vivo (*1*), prevents the access to brain of even relatively small peptide-based drugs. Therefore, despite the potentially high receptor-binding affinity and metabolic stability of neuropeptide analogues, centrally mediated effects are difficult to elicit after systemic administration of the peptide (*2*). In the case of opioid peptides, this could be recently quantitatively confirmed with a pharmacokinetic study using DALDA, a metabolically stable dermorphin analogue tetrapeptide (*3*). Clinically useful neuropeptide pharmaceuticals may be developed using suitable brain drug delivery strategies. One delivery approach is the use of "chimeric peptides", which consist of the nontransportable neuropeptide pharmaceutical coupled to a transport vector (*2*). A model vector is the OX26 monoclonal antibody against the rat transferrin receptor, which undergoes receptor-mediated transcytosis through the BBB in vivo (*2*). The successful practical implementation of the chimeric peptide approach requires optimization in three inter-related areas. First, vectors with high BBB transcytosis rates must be developed. Second, a linker strategy must be applied, which provides reversible high yield coupling of different peptide ligands to the vector. These goals are achieved with the use of avidin–biotin technology and the production of avidin–vector conjugates and biotinylated peptide therapeutics (*4*). Recent studies have shown that optimal plasma pharmacokinetics of avidin–vector conjugates is obtained with the use of neutral avidin (NLA) (*5*). Another requirement with the avidin–biotin drug delivery approach is the use of a peptide ligand that is monobiotinylated. Higher degrees of biotinylation lead to the formation of aggregates owing to the multivalent binding of biotin by avidin. Third, the biotinylated peptide therapeutic must retain biologic activity following cleavage from the transport vector within the brain.

Progress toward achieving these goals has recently been demonstrated for a vasoactive intestinal peptide (VIP) analogue (*6*) and the opioid peptide, DALDA (*7*). In those studies, a cleavable biotin linker containing a disulfide bridge was used, which theoretically allows for release of the peptide moiety from the chimeric peptide following its transport into brain tissue. However, at present, at least three issues remain unresolved regarding the method of linking the peptide therapeutic to the avidin–vector conjugate. The first issue is whether a cleavable (e.g., disulfide) or noncleavable (e.g., amide) linkage should be used for attachment of the biotin moiety to the peptide. A noncleavable linker could be used if the peptide is biologically active when attached to the avidin–vector conjugate. Second, if a cleavable linkage is used, this bond must be stable in plasma in the circulation in vivo, stable during transit through the brain capillary endothelial cell, but rapidly cleaved in brain in vivo to release in biologically active form the

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"desbiotinylated" peptide from the avidin–vector conjugate. Third, if the cleavable linker is a disulfide bond, then the cleaved peptide therapeutic will invariably carry an added free thiol group generated by the cleavage of the disulfide group, and it is necessary to show reversibility of pharmacologic action in brain of the peptide. It is possible that thiolated peptides may form a disulfide linkage with the receptor, which could result in irreversible activation of the peptide receptor (*8*). These three issues are addressed in the present study, which uses [Lys^7]dermorphin, abbreviated K7DA, which has structural features similar to DALDA (*9*), but is intrinsically more potent with respect to binding to the μ-opioid peptide receptor (*10*). The K7DA peptide is conjugated with both cleavable and noncleavable biotin analogues, and the activity of the cleavable analogue, designated bio-SS-K7DA, versus the noncleavable analogue, designated bio-XX-K7DA, is investigated with opioid peptide radioreceptor assays. Second, the stability of the disulfide linker in plasma and brain in vivo and in brain capillary endothelial cells is evaluated using HPLC analysis of brain extracts. Third, the reversibility of the antinociceptive action of the thiolated K7DA in brain in vivo is shown using tail-flick analgesia assays following intracerebroventricular (icv) administration of opioid chimeric peptides or the μ-opioid antagonist, naloxone.

EXPERIMENTAL PROCEDURES

Materials. Na^{125}I was supplied by Amersham (Arlington Heights, IL). DAGO, [^{3}H]DAGO (specific activity, 38.4 Ci/mmol), DPDPE, and [^{3}H]DPDPE (specific activity, 27.38 Ci/mmol) were provided by the National Institute of Drug Abuse Research Technology Branch (Rockville, MD). [^{14}C]Sucrose (specific activity, 632 mCi/mmol) was obtained from NEN Dupont (Wilmington, DE). Sulfosuccinimidyl 2-(biotinamidoethyl)-1,3′-dithiopropionate (NHS-SS-biotin), 2-iminothiolane (Traut's reagent), and m-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS) was supplied by Pierce Chemical (Rockford, IL). Biotin-XX-NHS was supplied by CalBiochem (San Diego, CA), where XX = bis(aminohexanoyl) spacer arm and NHS = *N*-hydroxysuccinimide. Acetonitrile was obtained from Fisher Scientific (Tustin, CA). Chloramine T was purchased from MCB Reagents (Cincinnati, OH). Neutralite avidin (NLA) was supplied by Accurate Chemicals (Westbury, NY). Recombinant streptavidin (SA) and all other reagents were obtained from Sigma (St. Louis, MO). Vydac C_4 (10 × 250 mm) reversed-phase HPLC columns were obtained from the Separations Group (Hesperia, CA), and Sephacryl S300HR was from Pharmacia (Piscataway, NJ). G-25 Quick Spin columns were obtained from Boehringer Mannheim (Indiana, IN). TSK-gel G2000 SW_{XL} HPLC columns (7.8 × 300 mm) were obtained from TosoHaas (Montgomeryville, PA). The 22-gauge guide cannula, 28-gauge dummy cannula, and 28-gauge injection cannula for icv injections were supplied by Plastics One (Roanoke, VA). The Model 33 tail-flick analgesia meter was obtained from IITC Life Sciences (Woodland Hills, CA). Male Sprague–Dawley rats (220–270 g body weight) were supplied by Harlan Sprague–Dawley (Indianapolis, IN).

Synthesis and Biotinylation of the Dermorphin Analogue. Lys^7-dermorphin analogue (K7DA) was synthesized in its α-*N*-fmoc-Tyr^1 protected form by the Peptide Synthesis Facility, Department of Biological Chemistry (University of California, Los Angeles). The solid phase peptide synthesis and subsequent HPLC purification were performed as described previously (*7*). Fmoc-K7DA was then biotinylated with either NHS-SS-biotin or NHS-XX-biotin as described (*7*). Briefly, 500 μg of the fmoc-peptide were dissolved in 400 μL of dimethyl sulfoxide, and 600 μL of 0.05 M $NaHCO_3$ (pH 8.3) was added. A 3- to 6-fold molar excess of the biotinylating reagent (NHS-SS-biotin or NHS-XX-biotin) in 1 mL of 0.05 M $NaHCO_3$ was added. After 60 min at room temperature, the reaction was stopped by the addition of 100 μL of trifluouroacetic acid (TFA) and 500 μL of ACN. After HPLC purification, the N-terminal fmoc group was removed, and the bio-SS- or bio-XX-K7DA was again HPLC purified as described (*7*). In vitro cleavage of bio-SS-K7DA to obtain desbiotinylated K7DA (desbio-K7DA) was performed with 50 mM dithiothreitol (DTT) in 50 mM phosphate buffer (pH 7.5) for 1 h at room temperature. Bio-SS-K7DA and desbio-K7DA were analyzed by secondary ion mass spectrometry (SIMS), as described (*7*). Peptide amounts after HPLC purification were quantitated with the BCA reagent (Pierce Chemical Co., Rockford, IL).

Synthesis of Avidin–OX26 Conjugates. A conjugate of the antitransferrin receptor monoclonal antibody, OX26, and either neutralite avidin (NLA) or streptavidin (SA) was prepared as described previously (*4, 5*). Briefly, OX26 was thiolated with Traut's reagent, and NLA or SA was activated with MBS. The thiolated OX26 and activated NLA or SA were then mixed, and the conjugate was purified over a Sephacryl S300 HR column (*5*). The fractions corresponding to the conjugate of NLA–OX26 or SA–OX26 were separated from unconjugated NLA or SA and from high molecular weight aggregates (*5*). The number of biotin binding sites per OX26 conjugate was determined as described previously (*5*) and was 4.2 ± 0.1 and 3.3 ± 0.3 for NLA–OX26 and SA–OX26, respectively.

In Vitro and in Vivo Testing of Opioid Receptor Affinity. Opioid radioreceptor assays (RRA) with rat brain membranes (*11*) were performed, as described in ref *7*. Tail-flick analgesia measurements in rats were performed after icv injections of the following peptides: K7DA, bio-XX-K7DA, bio-XX-K7DA bound to NLA-OX26, bio-SS-K7DA bound to NLA-OX26, and desbio-K7DA. The peptides were dissolved in 5 mM Na phosphate-buffered saline containing 0.05% Tween-20. The injection volume was 20 μL. Naloxone reversibility of the analgesia was tested by an icv injection of 20 μg/20 μL naloxone in saline. The stereotaxic implantations in the lateral ventricle and the tail-flick analgesia testing was performed as described (*7*). Base-line latency was 3–4 s, and the cutoff time was set at 10 s.

Radiolabeling of bio-SS-K7DA. A 1–2 μg (0.8–1.6 nmol) portion of the peptide was labeled with 2 mCi Na^{125}I by the addition of 1.4–2.5 μg of chloramine T. The final reaction volume was 32 μL in 0.1 M Na phosphate buffer, pH 7.4. The reaction was stopped after 30–60 s at room temperature by the addition of 1.9–6.2 μg of sodium metabisulfite. The iodinated bio-SS-K7DA was then purified with one of two approaches. In the first approach, 0.4 nmol of the ^{125}I-bio-SS-K7DA was mixed with 50 μL of NLA-OX26 (1.74 mg/mL) in 5 mM PBS/0.05% Tween-20 followed by incubation for 15 min at room temperature. The ^{125}I-bio-SS-K7DA bound to the NLA-OX26 was purified from free iodine over Quick-Spin G-25 Sephadex columns (sample volume 45 μL each), which had been equilibrated with 10 mM PBS/0.05% Tween-20. The combined eluant volume from two Quick Spin columns was 120 μL. In the second purification approach, the ^{125}I-bio-SS-K7DA mixture was acidified by the addition of 1 mL of 1% trifluouroacetic acid (TFA), and the mixture was applied to an activated C18 SepPak cartridge. The extraction cartridge was washed with 10 mL of 0.1% TFA, and the iodinated peptide was eluted

with 5 mL of 60% acetonitrile in 0.1% TFA. The acetonitrile was removed by evaporation, and the final mixture was made 0.05 M Na_2HPO_4, pH = 7.4, 0.05% sodium azide, and was stored at 4 °C. The specific activity of the final product was 0.57 mCi/nmol with a trichloroacetic acid (TCA) precipitability of 95%.

Internal Carotid Artery Perfusion and Chromatographic Analysis. The in vivo brain uptake of the radiolabeled bio-SS-K7DA bound to the NLA-OX26 was studied with the internal carotid artery perfusion technique (*6*). Rats were anesthetized with ketamine (100 mg/kg) and xylazine (2 mg/kg) intraperitoneally, and the occipital, superior thyroid, and pterygopalatine arteries were closed by electrocoagulation. The external carotid artery was cannulated with PE10 tubing and before starting the perfusion of the ipsilateral brain hemisphere, the common carotid artery was completely closed by ligation. The perfusate contained ^{125}I-bio-SS-K7DA (3 μCi/mL or 10 nM) in Krebs–Henseleit buffer, 1% bovine serum albumin, and 10 nM NLA–OX26 and was oxygenated with 95% O_2 and 5% CO_2 and perfused at a rate of 1.25 mL/min for 10 min. At the end of the perfusion, the animal was decapitated, and the brain was homogenized on ice in 6 vol of homogenization buffer [0.1 M Na phosphate (pH = 7.0), 0.5 M NaCl, 0.25% bovine serum albumin (BSA), and 0.05% Tween-20] with a Polytron homogenizer for 10 s followed by sonication for 10 s. The homogenate was centrifuged at 50 000*g* for 60 min at 4 °C. An aliquot of the supernatant was counted for total radioactivity and a 250 μL aliquot was injected onto a TSK-gel G2000 SW_{XL} gel filtration column (7.8 × 300 mm). The column was eluted at 0.5 mL/min with the same buffer used for homogenization, and 1 min fractions were collected for 40 min and the fractions were then counted for ^{125}I-radioactivity. The void and salt volumes of the columns are 6 and 14 mL, respectively. In control experiments, the same HPLC analysis was performed with tracer in buffer alone, with tracer preequilibrated for 60 min at room temperature with 20 mM DTT, and tracer added to fresh rat brain in ice cold homogenization buffer, which was then homogenized as described above prior to HPLC injection. In all the experiments, the tracer is ^{125}I-bio-SS-K7DA conjugated to the NLA–OX26 complex.

Brain Capillary Uptake and Chromatographic Analysis. The cleavage of the ^{125}I-bio-SS-K7DA conjugated to the NLA–OX26 complex by isolated bovine brain capillaries at 37 °C was investigated. For these experiments, brain capillaries were isolated from fresh bovine brain using the mechanical homogenization technique described previously (*12*). Bovine brain capillaries (equivalent to 200 μg of capillary protein) were suspended for 2 or 20 min at 37 °C in 200 μL of 10 mM Hepes/0.15 M NaCl/pH = 7.4 containing 0.1% BSA and 0.1 μCi (0.3 pmol) of ^{125}I-bio-SS-K7DA conjugated to 1 μg (5 pmol) of NLA–OX26. Control experiments included 20 min incubations of the ^{125}I-bio-SS-K7DA/NLA–OX26 without microvessels, but with 0 or 500 mM DTT. The ^{125}I-bio-SS-K7DA was purified by C18 reversed-phase HPLC prior to the experiment. After the 2 or 20 min incubation, the tubes were placed on ice and centrifuged at 10 000*g* for 60 s at 4 °C. The supernatant was removed for direct HPLC analysis. The capillary pellet was drained of supernatant and was suspended in 0.1 M Na phosphate (pH = 7.4), 0.5 M NaCl, 1 mM EDTA, and 0.05% Tween-20. The capillary suspension was sonicated for 20 s on ice and centrifuged at 10 000*g* for 10 min at 4 °C. Ten μL aliquots of the capillary supernatant were then counted for radioactivity, and the remainder was injected onto the TSK-gel filtration HPLC column described above.

Intravenous Injection and Chromatographic Analysis. The relative stability of the disulfide linker in plasma and brain in vivo was assessed by intravenous injection of the ^{125}I-bio-SS-K7DA conjugated to the SA–OX26 complex. In these experiments, rats (270 g) were anesthetized with ketamine/xylazine as described above, and a 0.2 mL injection volume of Ringer's solution buffered with 10 mM Hepes was injected. The injection solution contained 0.1% rat serum albumin (RSA), 20 μCi of ^{125}I-bio-SS-K7DA, and 20 μg of SA–OX26. At 60 min after injection, the animals were sacrificed by decapitation, and plasma and brain were obtained. A brain hemisphere was homogenized with a Polytron homogenizer in three volumes of 0.01 M PBS, pH = 7.4, 0.05% Tween-20, followed by centrifugation at 20 000*g* for 30 min at 4 °C. The supernatant was then injected onto the TSK-gel filtration column as described above; 80 μL of the hemisphere supernatant was taken from each of the three rats analyzed in triplicate and pooled prior to injection onto the HPLC column. Similarly, 80 μL of the 60 min plasma was obtained from each rat, pooled, and injected onto the HPLC column, which was eluted in 0.1 M PBS, pH = 7.4, 0.05% Tween-20, as described above.

RESULTS

Fmoc-K7DA was efficiently biotinylated with NHS-SS-biotin, as more than 80% of the peptide eluted as bio-fmoc-K7DA from the HPLC column (Figure 1). Alkaline deprotection and HPLC purification of bio-SS-K7DA yielded an overall recovery of 61% of the original peptide. When the biotin-XX-NHS ester was used for biotinylation, the overall recovery of biotinylation and deprotection exceeded 90%. The bio-SS-K7DA was converted into desbio-K7DA with dithiothreitol (DTT) treatment, and this conversion was complete as shown in Figure 1. The structure of the bio-SS-K7DA and the desbio-K7DA was confirmed with secondary ion mass spectrometry (SIMS), and the experimentally determined molecular masses were within less than one mass unit of the formula weights of the compound (Figure 2).

The biologic activity of the desbio-K7DA was assessed with a μ-opioid peptide radioreceptor assay using [^{3}H]-DAGO as the μ-receptor-specific ligand. The binding isotherms for unlabeled DAGO, K7DA, and desbio-K7DA are shown in Figure 3A, and the K_i values for each of these compounds are given in Table 1. The K_i value for K7DA was 0.62 ± 0.14 nM, and this was not significantly different from the K_D of DAGO, 0.58 ± 0.08 nM (Table 1). The desbio-K7DA had an affinity for the μ-receptor approximately 50% less than that of the parent compound with a K_i of 1.24 ± 0.24 nM. The displacement of [^{3}H]DAGO from the μ-receptor by bio-XX-K7DA in either its free form or conjugated to the NLA–OX26 vector is shown in panel Figure 3B. The K_i of the bio-XX-K7DA was 1.5 ± 0.27 nM (Table 1), but the K_i of this analogue bound to the NLA–OX26 vector was immeasurably high (Figure 3, Table 1).

The high analgesic potency of K7DA is shown by the tail-flick analgesia experiments following icv injection of 0.01, 0.03, and 0.10 nmol of K7DA (Figure 4A). Similarly, bio-XX-K7DA demonstrated a strong antinociceptive response following the icv injection of 0.1 nmol (Figure 4B). In parallel with the radioreceptor assay (Figure 3B), the binding of the bio-XX-K7DA to the NLA–OX26 conjugate resulted in a more than 90% inhibition of the antinociceptive response of the biotinylated peptide (Figure 4B). Similarly, binding of the bio-SS-K7DA to the NLA–OX26 conjugate resulted in suppression of the

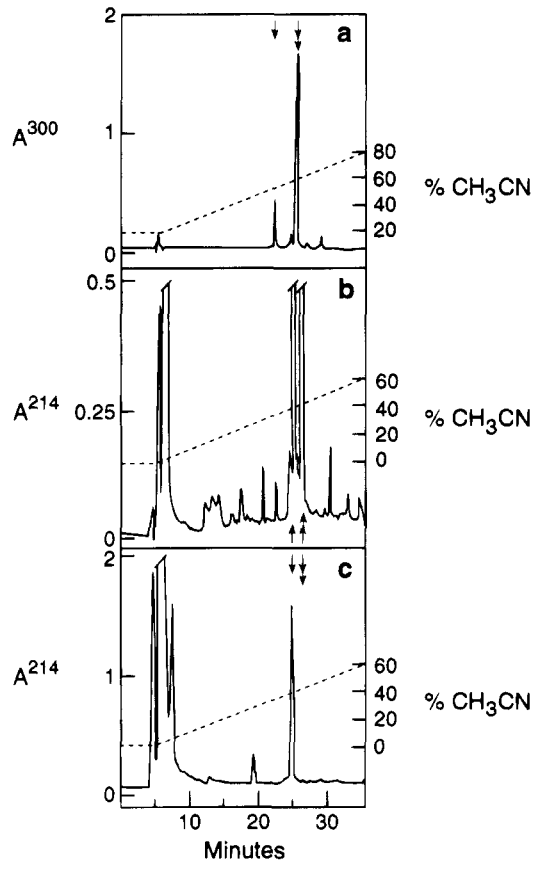

Figure 1. Reversed-phase HPLC on a Vydac C_4 column of bio-SS-fmoc-K7DA (A), bio-SS-K7DA following deprotection of the fmoc group (B), and of desbio-K7DA after cleavage by DTT (C). The elution was monitored at $\lambda = 300$ nm (A), which detects the fmoc group, or 214 nm (B, C). The broken lines indicate the gradient of acetonitrile in 0.1% trifluouroacetic acid. The single arrow in A indicates the retention time of the nonbiotinylated fmoc-K7DA. The single arrow in B denotes a reagent peak (no measurable peptide content), and the double arrows in B and C indicate the retention time of bio-SS-K7DA.

analgesic response (Figure 4C). However, when the cleavable linker was used, the analgesic potency of the vector-bound peptide was restored by cleavage under mild reducing conditions, e.g., the addition of 0.5 mM cysteine (Cys) to the icv injection solution. Doses of 0.1 and 0.3 nmol of bio-SS-K7DA bound to the NLA-OX26 conjugate showed a dose-related, yet delayed, analgesic response (Figure 4C), indicating a slow in vivo release of the desbio-K7DA from the chimeric peptide after icv injection.

The administration of 0.1 nmol of desbio-K7DA elicited a maximum analgesic response (Figure 4D). The μ-opioid nature of the analgesic effect could be demonstrated by the immediate reversibility of the analgesia by the icv injection of 25 μg of naloxone, as indicated by the arrows in panel 4D. The potency and long duration of the desbio-K7DA was evident from the gradual escape from naloxone antagonism over the next 30 min. The full naloxone effect could be restored by a repeated icv administration of an additional 25 μg dose of the antagonist. The experiments shown in Figure 4D also demonstrate that the NLA–OX26, added at a 1:1 molar ratio, did not interfere with the analgesic effect of the desbio-K7DA. In other experiments, the separate administration of 20 μL of 0.5 mM L-cysteine (equivalent to 10 nmol) had no analgesic effect following icv administration.

The selective cleavage of the disulfide linker in brain, but not in plasma, was demonstrated by intravenous injection of ^{125}I-bio-SS-K7DA bound to the NLA–OX26 conjugate. As shown in Figure 5A, the ^{125}I-bio-SS-K7Da in plasma obtained 60 min after iv administration eluted from the HPLC gel filtration column with the NLA–OX26 conjugate at 7 mL. Conversely, HPLC analysis of an extract of rat brain obtained 60 min after iv injection of the chimeric peptide demonstrated that there was nearly complete conversion of the radiolabeled chimeric peptide to the free desbio-K7DA or its metabolites (Figure 5B).

It is conceivable that the radiolabeled chimeric peptide was cleaved in the plasma compartment followed by brain uptake of low molecular weight metabolites. Therefore, the in vivo cleavage of the disulfide bond by rat brain was investigated with an internal carotid artery perfusion technique. Prior to perfusion, the ^{125}I-bio-SS-K7DA/NLA–OX26 conjugate eluted at a column volume of 7 mL (Figure 6A). No radioactivity eluted in the fractions where the free ^{125}I-bio-SS-K7DA or free iodine would be expected (Figure 6A), indicating an efficient conjugation of the biotinylated peptide to the NLA–OX26 vector. The in vitro cleavability of the chimeric peptide at the disulfide bridge was shown by pre-incubation of the labeled chimeric peptide with 20 mM DTT for 60 min at room temperature followed by gel filtration HPLC. As shown in Figure 6B, the radioactivity peak completely shifted from the elution volume (7 mL) of the NLA–OX26 to the elution volume (14 mL) corresponding to the unconjugated peptide migrating in the salt volume of the column. The ^{125}I-bio-SS-K7DA/NLA–OX26 was perfused into the internal carotid artery for 10 min, and the labeled chimeric opioid peptide reached a distribution volume of 30 ± 5 μL/g, a volume that is more than 3-fold greater than the plasma volume, 8 μL/g (*5*). When the brain homogenate was cleared by centrifugation and subjected to gel filtration HPLC analysis, cleavage within the 10 min brain perfusion was observed (Figure 6C). The cleavage had occurred in vivo, since a control experiment, where the tracer was added to fresh rat brain tissue prior to the same homogenization and chromatography procedure, did not reveal any cleavage of the disulfide bond, as shown in Figure 6D.

In order to determine whether brain microvessels are capable of cleaving the chimeric peptide, the ^{125}I-bio-SS-K7DA/NLA-OX26 was also incubated with isolated bovine brain capillaries at 37 °C for 2–20 min. HPLC analysis of the medium (Figure 7A) after a 20 min incubation showed minimal conversion of the ^{125}I-bio-SS-K7DA into its nonconjugated form (Figure 7A). HPLC analysis of the 20 min microvessel pellet showed that the majority of the ^{125}I-bio-SS-K7DA remained conjugated to the NLA-OX26 vector (Figure 7B). There was no difference between the chromatograms obtained at 2 or 20 min of incubation.

DISCUSSION

The results of the present studies are consistent with the following conclusions. First, a cleavable, e.g., disulfide, linker joining the opioid peptide (K7DA) to the transport vector (OX26 monoclonal antibody) is required over a noncleavable (e.g., amide) linker, because the opioid peptide has a marked reduction in biologic activity when directly conjugated to the transport vector (Figures 3 and 4). Second, a disulfide linker demonstrates optimal characteristics with stability in plasma and brain endothelial cells, but rapid cleavage in brain in vivo (Figures 5–7). Third, cleavage of the opioid peptide from the transport vector results in thiolation of the opioid peptide (Figure 2), yet the thiolated K7DA has a high degree of

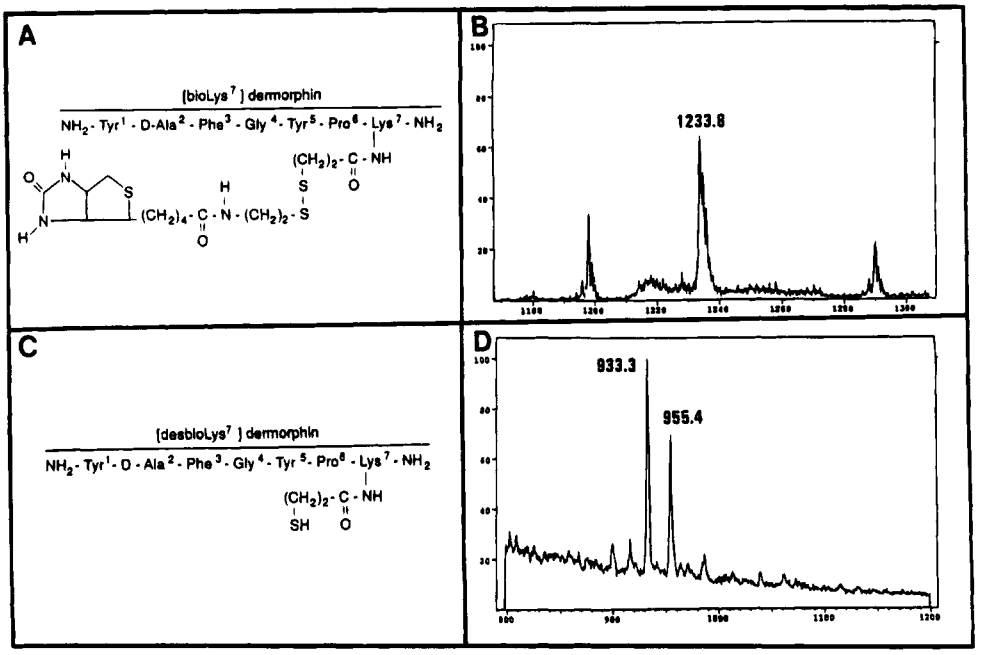

Figure 2. Structural formulas of (A) bio-SS-K7DA (also called bio-Lys^7-dermorphin) and (C) desbio-K7DA (also called desbio-Lys^7-dermorphin). Panels B and D show the corresponding molecular weights as measured by secondary ion mass spectrometry (SIMS). The formula weights are 1233.62 (bio-SS-K7DA) and 932.17 (bio-K7DA). The mass peak at 955.4 in D corresponds to an Na-adduct of desbio-K7DA.

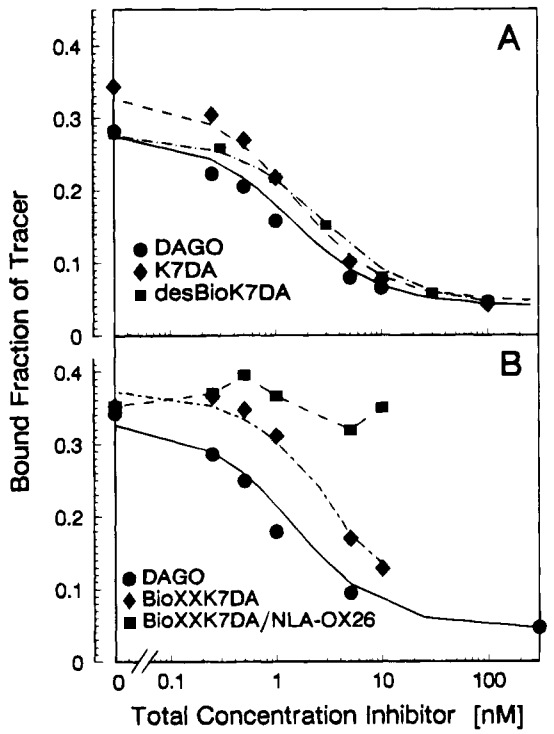

Figure 3. Competition curves of radioreceptor assays with ^{3}H-DAGO. A and B show the results of separate experiments. The data points are means of duplicates, where the coefficient of variation was less than 5%. The curves are best-fits using least-squares nonlinear regression analysis (except for bio-XX-K7DA/NLA-OX26). The corresponding inhibitor constants (K_i) are given in Table 1. The concentration of NLA–OX26 was 20 μg/mL (93 nM) and was in excess of the bio-XX-K7DA, which ranged from 0.25 to 10 nM (for the Bio-XX-K7DA/NLA–OX26 curve).

intrinsic receptor activity (Figures 3 and 4) and results in the pharmacologic activation of the μ-opioid peptide receptor that is reversible with naloxone treatment (Figure 4).

Table 1. Radioreceptor Assay with Synaptosomes from Rat Brain[a,b]

ligand	B_{max} (fmol mg$_p^{-1}$)	K_i (nM)
DAGO	105 ● 11	0.58 ± 0.08
K7DA		0.62 ± 0.14
desbio-SS-K7DA		1.24 ± 0.24
bio-XX-K7DA		1.59 ± 0.27
bio-XX-K7DA/NLA–OX26		>10

[a] Nonspecific binding = 4.2 ± 0.5%. [b] Parameters were obtained by least-quares nonlinear regression from the competition data shown in Figure 3. [^{3}H]DAGO was used as the labeled μ-specific ligand at a concentration of 0.5 nM.

The noncleavable biotin linker generated with the use of NHS-XX-biotin introduces a 14-atom spacer between the opioid peptide and the biotin moiety. Previous studies have shown that biotinylated β-endorphin derivatives bind the μ-opioid peptide receptor while conjugated to avidin (*13*). However, the present studies demonstrate that when the avidin is in turn conjugated through a thiol–ether linkage to a 150 000 Da monoclonal antibody, there is a marked reduction in the affinity of the opioid peptide for the μ-receptor based on either radioreceptor assays (Figure 3, Table 1) or analgesia testing following icv administration (Figure 4B). These studies indicate the necessity of choosing a cleavable linker joining the peptide therapeutic to the transport vector. Disulfide linkages are preferred because of the relative stability of this linkage in plasma (*14*) and extracellular fluids such as CSF (*15*).

The stability of the disulfide linker joining the peptide therapeutic with the NLA–OX26 transport vector in plasma and in endothelial cells is demonstrated in Figures 5 and 7, respectively. Previous studies have shown that the disulfide linker is rapidly cleaved by homogenate of rat brain in vitro at 37 °C (*16*). The present studies extend these results to the in vivo state using internal carotid artery perfusion and show that the disulfide linker is approximately 40% cleaved within 10 min of an internal carotid artery perfusion. This duration of perfusion allows the conjugate to readily access

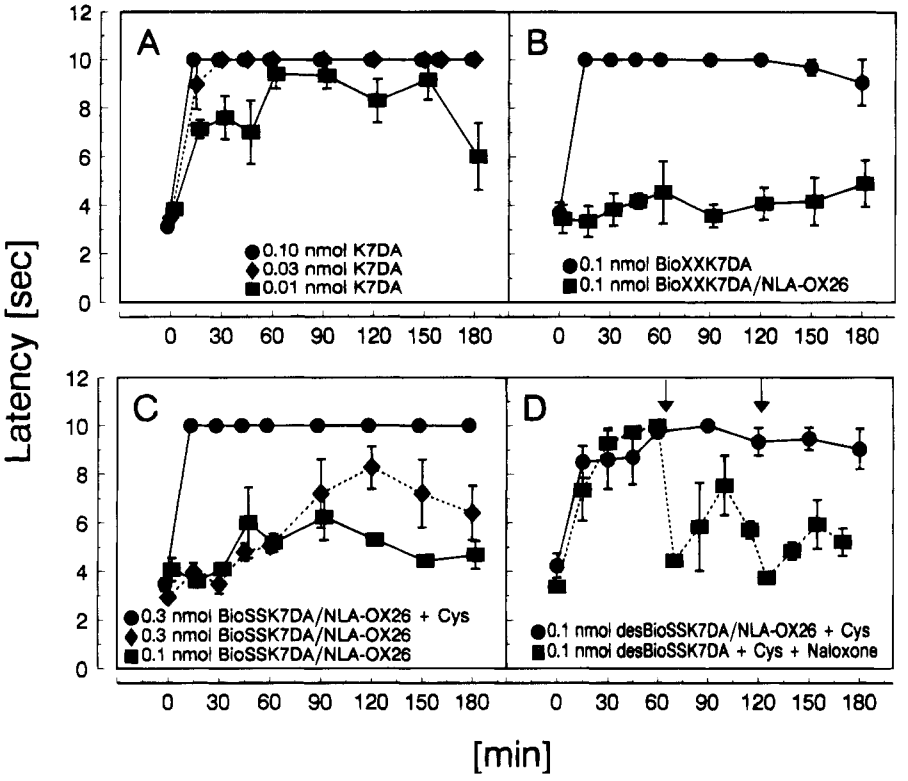

Figure 4. Tailflick analgesia measurements after icv administration of the peptide. Doses are given in the insets. A shows a dose–response curve of K7DA. B compares the analgesic effect of bio-XX-K7DA with or without binding to NLA–OX26. C shows the effect of two different doses of bio-SS-K7DA/NLA–OX26 and the effect of precleavage of the chimeric peptide with 0.5 mM cysteine (Cys). D shows the naloxone reversibility of desbio-SS-K7DA and the effect of desbio-SS-K7DA in the presence of the vector, NLA–OX26. The arrows in D indicate the time of naloxone administration. $n = 3$ for all experiments, data points are mean ± SE.

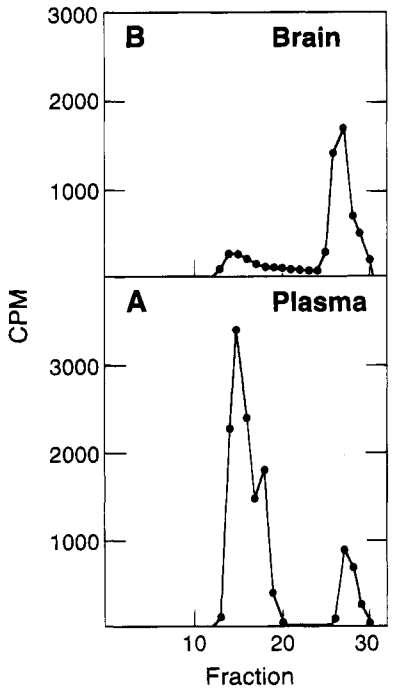

Figure 5. Gel filtration HPLC of plasma (A) and brain homogenate (B) obtained 60 min after intravenous injection of ^{125}I-bio-SS-K7DA/NLA–OX26. Aliquots of plasma or brain homogenate were pooled from three rats performed in triplicate and injected onto the TSK-gel filtration column (7.8 × 300 mm). The column was eluted at 0.5 mL/min, and 0.5 mL fractions were collected and counted for ^{125}I-radioactivity (cpm = counts per minute).

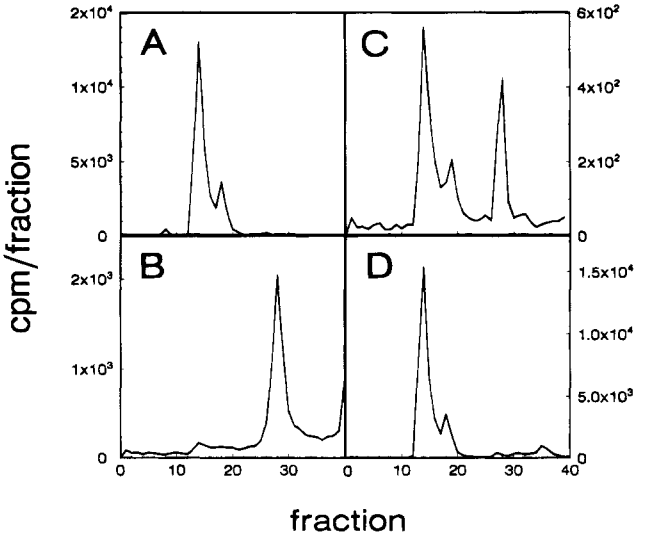

Figure 6. Gel filtration HPLC of (A) ^{125}I-bio-SS-K7DA/NLA–OX26 and (B) same tracer after treatment with 20 mM DTT. (C) Chromatogram of supernatant of brain homogenate following a 10 min brain perfusion. D is the control, where the tracer was added to fresh brain tissue in ice cold homogenization buffer before homogenization. Fractions = 0.5 mL.

brain postvascular spaces. Previous studies have shown that about 45% of the peptide/vector complex that is taken up by the brain has distributed to the postvascular compartment (*6*). Longer experimental time periods lead to greater disulfide cleavage. For example, 60 min after an intravenous injection, the amount of opioid peptide bound to the transport vector within brain is minimal (Figure 6B).

The stability of the disulfide bond in brain endothelial cells (Figure 7) suggests the chimeric peptide is retained within an endosomal compartment of the microvessels and is not transferred to the endothelial cytosol, where

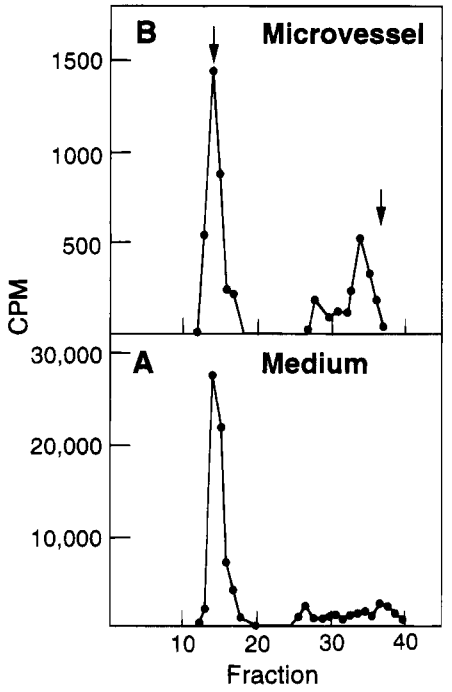

Figure 7. Gel filtration HPLC of ^{125}I-bio-SS-K7DA/NLA–OX26 incubated for 20 min with isolated bovine brain capillaries at 37 °C. A is the chromatogram of the medium obtained after centrifugation of the capillaries and B is the chromatogram of the microvessel pellet homogenate. The left-hand and right-hand arrows in panel B point to the elution volume of the ^{125}I-bio-SS-K7DA conjugated to the NLA–OX26 and after release from the NLA–OX26 with DTT treatment, respectively.

disulfide bonds are rapidly cleaved (*17*). These results are consistent with previous studies showing that disulfide cleavage does not occur in endosomes (*18*), but is primarily a concomitant of entry into the cytosolic space owing to the very high ratio of glutathione to oxidized glutathione in this compartment (*19*). The stability of the disulfide linkage within the endothelial compartment is believed to be an important requirement allowing efficacy of chimeric peptide pharmaceuticals in brain. If the disulfide linker was stable in plasma but was reduced in brain capillary endothelium, then peptide therapeutic may not be enabled to undergo exocytosis across the ablumenal membrane of the brain capillary endothelium and enter brain interstitial space. Previous studies with conjugates of the OX26 monoclonal antibody and 5 nm gold particles have shown that the OX26 conjugate traverses the endothelial compartment within endosomal structures and does not enter into the endothelial cytosol (*20*). Another possible site of disulfide cleavage is at the plasma membrane surface, and recent studies have shown that disulfide cleavage may occur at the plasma membrane owing to cell surface bound protein disulfide isomerase (PDI) (*21*). However, the present studies with isolated bovine brain capillaries (Figure 7) indicate there is not rapid cleavage of the disulfide linker at the surface of brain capillaries. Both luminal and abluminal membranes of the capillary endothelium are exposed in the isolated microvessel experiments. Prior work using dye-coated microspheres has shown the microvessels are patent and the luminal space is rapidly accessed by diffusion (*22*).

The cleavage of the opioid peptide from the transport vector via reduction of the disulfide bridge results in the release of a thiolated form of the peptide therapeutic, called desbio-K7DA. The structure for desbio-K7DA is given in Figure 2C and shows a mercaptopropionate group on the Lys^7 moiety of the opioid peptide. The high biologic activity of the desbio-K7DA in either the radioreceptor assay or in the icv analgesia testing (Figures 3 and 4) indicates the thiol moiety on the K7DA at the Lys^7 position does not compromise interaction of the opioid peptide with the μ-receptor. The in vitro radioreceptor assays with the desbio-K7DA correspond to previous studies with desbio-DALDA showing high affinity of the thiolated or desbio-DALDA with the μ-opioid peptide receptor (*7*). The present studies extend the radioreceptor assays to the in vivo state and show that the cleavage of the bio-SS-K7DA from the NLA–OX26 vector with cysteine results in a marked restoration of opioid peptide-mediated analgesia, owing to release of the opioid peptide from the NLA–OX26 vector (Figure 4C). In addition to retention of biologic activity of the opioid peptide following cleavage from the transport vector, it is also desirable to maintain reversibility of biologic activity of the thiolated opioid peptide. Previous studies have shown that thiolated enkephalin derivatives covalently attach to the δ-opioid peptide receptor (*8*), resulting in sustained antagonism of analgesia mediated by δ-specific analogues (*23*). The present studies provide evidence that the thiolated or desbio-K7DA is not irreversibly attached to the μ-opioid peptide receptor, since the pharmacologic effect can be repeatedly reversed by the icv administration of naloxone (Figure 4D).

In summary, the present studies demonstrate the design features that must be incorporated in the linkage of opioid peptides to BBB transport vectors such as the OX26 monoclonal antibody. Owing to the intrinsically low efficiency of direct peptide conjugation to transport vectors using chemical coupling strategies (*2*), avidin–biotin technology was introduced to allow for high efficiency coupling of the peptide therapeutic to the transport vector, which consists of a stable thiol–ether conjugate of the OX26 monoclonal antibody and avidin. In forming biotinylated peptide derivatives that bind with high affinity to the NLA–OX26 conjugate, the following design features are required. First, the peptide must be monobiotinylated in order to prevent the formation of high molecular weight aggregates owing to the multivalent binding of biotin by avidin. Second, a cleavable, e.g., disulfide, linker joining the peptide therapeutic to the biotin moiety must be used owing to the lack of biologic activity of the peptide therapeutic when attached to the NLA–OX26 conjugate, which has a molecular weight of approximately 215 000 Da. Third, the cleavage of the disulfide linker must result in release of the opioid peptide in biologically active form. This requirement is achieved by selectively biotinylating the Lys^7 ϵ-amino group and maintaining a free amino terminus with the use of fmoc-protected amino terminus on the opioid peptide (Figure 2A); the free amino terminus is required for opioid peptide activity (*24*). Fourth, the activation of the target receptor by the thiolated or desbio-peptide therapeutic must be reversible by an antagonist such as naloxone, indicative of lack of covalent activation of the target receptor by the thiolated peptide therapeutic. Fifth, the peptide therapeutic must be metabolically stable and the stability of the K7DA is enhanced by the D-Ala^2 moiety (Figure 2), which blocks susceptibility of the peptide to aminopeptidase activity, an ecto-enzyme that is enriched in brain microvessels (*25*) and is naturally occurring in dermorphin peptides (*26*). The multiple design features that must be considered in developing an efficacious linker strategy underscore the complexities involved in the development of drug delivery paradigms and the need to achieve progress in three spheres simultaneously, namely the vector, the linker strategy, and retention of biologic activity of the therapeutic (*27*).

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