Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7114–7118, July 1994
Pharmacology

Glycopeptide enkephalin analogues produce analgesia in mice: Evidence for penetration of the blood–brain barrier

(drug delivery/opioid receptor/peptides/antinociception/glucosides)

ROBIN POLT*†, FRANK PORRECA‡, LAJOS Z. SZABÒ*, EDWARD J. BILSKY‡, PEG DAVIS‡, THOMAS J. ABBRUSCATO‡, THOMAS P. DAVIS‡, ROBERT HORVATH‡, HENRY I. YAMAMURA‡, AND VICTOR J. HRUBY*†

Departments of *Chemistry and ‡Pharmacology, University of Arizona, Tucson, AZ 85721

Communicated by Bruce Merrifield, March 25, 1994

ABSTRACT **Most peptides have not proved useful as neuroactive drugs because they are blocked by the blood–brain barrier and do not reach their receptors within the brain. Intraperitoneally administered L-serinyl β-D-glucoside analogues of [Met⁵]enkephalin (glycopeptides) have been shown to be transported across the blood–brain barrier to bind with targeted μ- and δ-opioid receptors in the mouse brain. The opioid nature of the binding has been demonstrated with intracerebroventricularly administered naloxone. Paradoxically, glucosylation decreases the lipophilicity of the peptides while promoting transport across the lipophilic endothelial layer. It is suggested that glucose transporter GLUT-1 is responsible for the transport of the peptide message. Profound and long-lasting analgesia has been observed in mice (tail-flick and hot-plate assays) with two of the glycopeptide analogues when administered intraperitoneally.**

Despite the fact that insulin has been used as a drug for many years, it has been widely accepted that peptide neuromodulators (hormones) fail to significantly affect their target cells within the brain when administered peripherally. The human brain is regulated by a large number of these peptide neuromodulators which are secreted by neurons. Drug design based on these natural peptide "messages" and their receptors within the brain has been the subject of intense study (ref. 1, Chapter 5; ref. 2), but the use of these peptide neuromodulators as pharmaceuticals is limited by the blood–brain barrier (BBB), which excludes most peptides from the brain (3, 4), although several enkephalin analogues have been shown to produce effects when administered peripherally (5). The vast potential for naturally occurring brain peptides and their unnatural analogues to alleviate diverse neurological conditions ranging from headaches and anxiety to Alzheimer disease and stroke is now widely recognized (ref. 1, p. 27). This potential can be realized only if effective penetration of the BBB can be achieved (6). Here we report our studies of O-linked glycopeptides related to [Met⁵]enkephalin.

Our rationale for the design of these drugs was to attach the hormone message (in this case, a stable, potent enkephalin analogue) to β-D-glucose, a substance which is actively transported into the brain. The hope was that the glucose moiety would function as a transport vector and that the conjugate would be transported across the BBB so that it could then bind with its endogenous opioid receptors. Glucose was chosen because it is the brain's principal nutrient. Other workers have used various transport vectors to penetrate the BBB. One approach involved the use of a biotinylated peptide which was conjugated to a monoclonal antibody to the transferrin receptor. This conjugate was shown to cross the BBB to produce central effects (7). An alternative, "lipophilic prodrug" strategy (8) has been used in which the lipophilicity of the peptide has been increased with lipid-soluble substituents which may then be cleaved by enzymes within the brain. This approach is not suitable for many peptides of interest, since increasing the lipophilicity of a peptide also decreases its solubility in serum (water). Ultimately, this lipophilic approach is self-limiting; in one case a nonaqueous solvent system must be used to administer the lipophile (9). Peptides also have been introduced directly via intracerebroventricular (i.c.v.) catheterization; it is obvious that new, noninvasive methods of administration are needed.

Receptor-mediated transport has been demonstrated for certain peptides [Tyr-W-MIF-1 (10), insulin (11), and transferrin (12), *inter alia*], and covalent attachment of nontransportable peptides to these transport vectors has been used as a strategy for transporting peptides into the brain. A number of polar metabolites required by the brain are actively transported into the brain, either by receptor-mediated endocytosis (13) or by gradient-driven cotransport systems (14). For example, researchers have demonstrated improved delivery of 3′-azido-3′-deoxythymidine (AZT) to the brain by using a glycosyl phosphotriester derivative of the drug, which is presumably transported by a nucleoside transporter (15).

In mammals β-D-glucose is rapidly transported into brain tissue by glucose transporters (e.g., GLUT-1; ref. 16) located in the endothelial cell membranes which compose the BBB. Both Lac permease, the H^+–β-galactoside cotransporter found in *Escherichia coli*, and SGLT-1, the intestinal Na^+–D-glucose cotransporter found in mammals, transport simple β-glucosides, although not nearly as well as their preferred substrates, β-D-lactose and β-D-glucose. Given the structural similarity of GLUT-1 with these transporters, it was reasoned that these transport systems might also transport biologically active peptides into the brain if the peptides were linked to β-D-glucose in the appropriate fashion. Thus, it was our hope that the peptide–β-D-glucoside linkage would not interfere with the binding of the glucose moiety to GLUT-1 or the binding of the peptide moiety to the opiate receptor. Scientists at Upjohn Laboratories have demonstrated that a glycopeptide renin inhibitor (N-linked glycopeptide in this case) has enhanced stability *in vivo*, presumably because of lower susceptibility to proteolytic enzymes (17). This "stability factor" may also play an important role in the transport of glycopeptide drugs. An α-D-glucoside of the clotting factor tuftsin has a prolonged lifetime in the bloodstream (18). None of these linkages would be expected to promote transport,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BBB, blood–brain barrier; DPDPE, [D-Pen²,⁵]enkephalin [where the D-penicillamine (D-Pen) residues are in disulfide linkage]; GPI, guinea pig ileum; MVD, mouse vas deferens; i.c.v., intracerebroventricular(ly); i.p., intraperitoneal(ly).

†To whom reprint requests should be addressed.

since the glucose transporter is specific for β-O-linked glucosides, not α-linked and not N-linked. Similarly, N-linked and O-acyl-linked enkephalin conjugates with glucose have not shown any BBB transport properties (19, 20).

Previously, two hydroxyproline (Hyp) O-linked glycopeptide enkephalin analogues have been synthesized [Tyr-D-Met-Gly-Phe-Hyp(β-Gal)-amide and Tyr-D-Met-Gly-Phe-Hyp(β-Glc)-amide] and tested for antinociception in rats (21). In that study, with centrally (i.c.v.) administered drug, the galactoside was >1700 times more potent than the glucoside, whereas the glucoside was slightly more potent outside the central nervous system. The authors suggested that diffusion was responsible for the different potencies. It could well be that diffusion of the glucoside is facilitated by the glucose transporter, which would reject the galactoside as a substrate, sequestering this glycoside within the brain.

There is now compelling evidence that morphine, one of the oldest drugs in the modern pharmacopoeia, can also be transported across the BBB in a physiologically active form as the 6-glucuronide metabolite (Fig. 1). Morphine 6-glucuronide, **A**, is 10–50 times more potent than morphine itself in producing analgesia (22, 23). Thus, morphine may function in part as a prodrug, with the 6-glucuronide producing a significant portion of the analgesic effect. β-D-Glucuronides **A** and **B** of morphine are formed *in vivo* by enzymatic processes in the liver. The naturally occurring enkephalins (**C**) and DPDPE (**D**) do not cross the BBB to any significant extent and are rapidly destroyed by peptidases in the bloodstream. While the potent synthetic δ-opioid agonist DPDPE, **D** (**10** in Table 1), a stabilized analogue, is resistant to enzymatic degradation, it too fails to significantly penetrate the BBB to produce analgesia (28).

MATERIALS AND METHODS

Enkephalin Analogues. The O-linked glycopeptides **1–7** (Table 1) were synthesized by glycosylation of serine Schiff-base esters with subsequent fluorenylmethoxycarbonyl-based solid-phase synthesis (29) and were purified by reversed-phase HPLC (2.2 cm × 25 cm Vydac C_8 column, 0–50% CH_3CN gradient vs. 0.1% CF_3SO_3H in water). The chemical structures were confirmed with 500-MHz ^1H NMR and fast-atom bombardment mass spectrometry. The related unglycosylated analogues **8** and **9** were synthesized, purified, and characterized in a similar fashion. Peptide **10**, DPDPE (1, 24, 30), is commercially available.

Brain and Serum Stability Incubations. Aliquots (180 μl) of resuspended, twice-washed 15% mouse brain homogenate or of mouse serum were placed into 1.5-ml centrifuge tubes. The tubes were prepared in triplicate for each time point (0, 10, 15, 30, 60, 120, and 240 min). An additional set of triplicate tubes were incubated with 50 mM Tris·HCl (pH 7.4) and served as buffer controls. Twenty microliters of 1 mM glycopeptide was dissolved in sterile water and added to each tube, which was agitated briefly (Vortex). Incubation was begun immediately at 37°C in a rolling water-bath incubator. Enzyme activity was terminated at the end of each incubation by adding 200 μl of CH_3CN and placing the tube on ice. Each tube was centrifuged at 3000 × *g*, and 300 μl of the supernatant was transferred to a clean 1.5-ml tube. An equal volume of H_2O was added, and the sample was mixed for HPLC analysis. A curvelinear gradient of 7–27% CH_3CN against 0.1 M sodium phosphate buffer (pH 2.4) was utilized, and baseline separation of glycopeptide **5** was achieved.

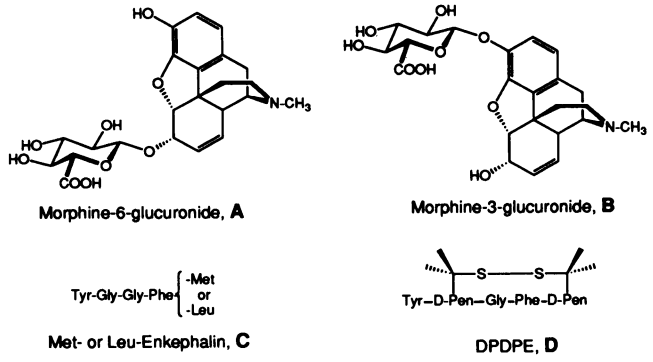


FIG. 1. Opioid receptor agonists. Compounds **A** and **B** are the morphine metabolites which are produced *in vivo* by enzymatic glycosylation of morphine in the liver. Metabolite **A** contributes significantly to the pharmacological effects of morphine (22, 23). The endogenous opioid ligands are the enkephalins, **C**. The [Met^5]-enkephalin analogue [D-Pen^{2,5}]enkephalin (DPDPE), **D**, is a potent opioid agonist selective for the δ-opioid receptor (1, 24–27).

Mouse Vas Deferens (MVD) and Guinea Pig Ileum (GPI) Bioassays. Electrically induced smooth muscle contraction of MVD and strips of GPI longitudal muscle–myenteric plexus was used as a bioassay (31). Percent inhibition was calculated as the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the agonist. IC_{50} values represent the mean of not less than four tissue samples. IC_{50} values, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation with a nonlinear least-squares method (32).

Radioligand Binding. Membranes were prepared from whole brains taken from adult male Sprague–Dawley rats (250–300 g) (Harlan–Sprague–Dawley). All radioligand displacement experiments were run against the ^3H-labeled ligands [D-Pen^2,Phe(*p*-Cl)^4,D-Pen^5]enkephalin (33) and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (25) as described previously. At least three experiments were done for each radioligand. Statistical comparisons between one- and two-site fits were made using the *F*-ratio test with a *P* value of 0.05 as the cutoff for significance (31). Data that were best fitted by a one-site model were reanalyzed with the logistic equation (34). Data obtained from independent measurements are presented as the arithmetic mean ± SEM.

Antinociception Studies. Male ICR mice (20–30 g) were used throughout these studies. They were housed in groups of four in Plexiglas boxes, maintained in a light- and temperature-controlled environment, with food and water available *ad libitum* until the time of antinociceptive testing. Glycopeptides **4** and **5** and peptides **8** and **9** were dissolved in distilled water (central administration), or physiological saline (peripheral administration). The i.c.v. administration was performed by the methods of Haley and McCormick (35) as modified by Porreca *et al.* (26). All testing was performed in accordance with the recommendations and policies of the International Association for the Study of Pain, the National Institutes of Health, and the University of Arizona guidelines for the care and use of laboratory animals.

Antinociception was assessed in mice by the warm water tail-flick assay and the hot-plate assay. In the tail-flick assay, tails were dipped in 55°C water, and the latency to a rapid tail flick was recorded with the baseline cutoff and the maximal possible latencies set at 5 sec and 15 sec, respectively. Percent antinociception was calculated as 100% × (test latency − control latency)/(15 sec − control latency). In the hot-plate assay, mice were placed on a 55°C surface, and the mean time to lick the back paws or escape jump was recorded. Percent antinociception was calculated as 100% × (test latency − control tatency)/(60 sec − control latency), with a cutoff latency of 20 sec (baseline).

Data are presented as the mean ± SEM for groups of 10 mice. Regression lines, ED_{50} and AD_{50} values, and their 95% confidence limits were calculated by using individual data

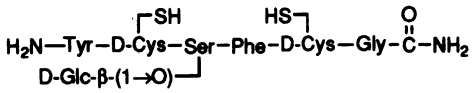
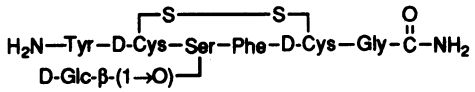
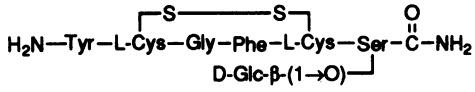
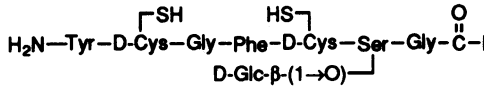
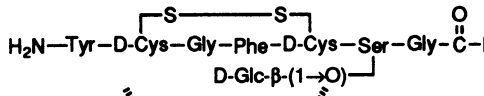
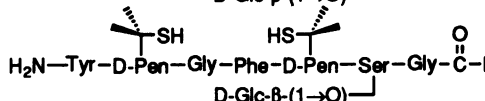
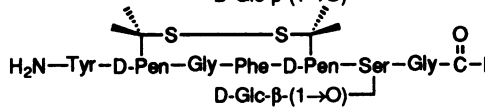
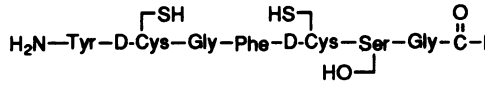
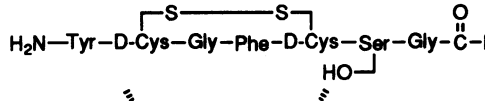
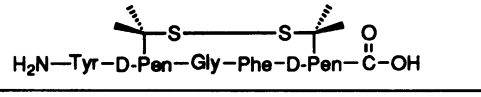
points with the computer program of Tallarida and Murray with procedure no. 8 (36).

RESULTS

Receptor Binding. Binding data for O-linked glycopeptide enkephalin analogues **1–7** (3-*O*-serinyl β-D-glucosides) and related unglycosylated analogues **8–10** are presented in Table 1. The binding constants (IC_{50} values) for the μ- and δ-opioid receptors for all of the opioid ligands were consistent with the bioactivity as determined by the MVD and GPI bioassays (1, 2, 28). From these data we conclude that all of the enkephalin analogues can bind to the opioid receptors of interest, except for compounds **1** and **2**. The poor opioid binding of **1** and **2** is easily understood upon consideration of the carbohydrate in the $i + 1$ position of the reverse turn (37), which normally "fits" into the receptor. This bulky, hydrophilic moiety presumably interferes with the opioid binding. In a sense, glycopeptides **1** and **2** correspond to the inactive morphine metabolite **B** (Fig. 1), in which the glucuronide prevents receptor–agonist interaction (receptor "fit").

Agonists related to DPDPE, **10**, were chosen for the "message segments" for several reasons. First, the receptor–ligand interactions (structure–activity relationships) for these compounds have been well studied (1, 2, 24–27, 30). Since our goal was to demonstrate the utilization of carbohydrate recognition and transport processes to deliver peptide "messages" to receptors within the brain, we did not wish to introduce any additional complications associated with novel peptide structures. The binding data displayed in Table 1 bear out the effectiveness of the message segments in binding to the opioid receptors. Second, the DPDPE-like peptide amides are quite potent opioid ligands and are much more stable in the presence of peptidases than the endogenous enkephalins, which are linear peptides. The stability of the cyclic glycopeptide **5** at 37°C was determined in mouse serum ($t_{1/2} \approx 80$ min) and in mouse brain ($t_{1/2} > 7$ hr) by HPLC analysis. Thus, behavioral effects (analgesia) were certain to be observed, provided that transport of the glycopeptides across the BBB could be achieved by the glucose transporter. Finally, the opioid receptors affected by these peptides are of great practical importance to medicine. It has been hypothesized (24, 38) that if an effective δ-selective agonist can be developed, such drugs might produce analgesia with limited tolerance and addiction liability. Glycopeptides **4** and **5** and the corresponding peptides **8** and **9**, which do not bear the

Table 1. Opioid binding activity *in vitro*

	Peptide ligand	IC_{50}, nM δ	μ	MVD	GPI
1		4000	2000	1900	18,000
2		3900	7700	520	3700
3		100	48	23	28
4		9.9	42	24	110
5		26	53	13	60
6		180	2400	680	125,000
7		85	48,000	560	40,000
8		4.2	20	6.8	25
9		6.1	30	5.5	26
10		6.5	11,000	4.1	7,300

Displacement of the δ-selective ^{3}H-labeled ligand [D-Pen^2, Phe(*p*-Cl)^4, D-Pen^5]enkephalin {[Phe(*p*-Cl)^4]DPDPE; ref. 33} and the μ-selective ^{3}H-labeled ligand D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (25) were used to characterize the opiate receptor binding activity (μ and δ) (27, 30) of ligands **1–9** in the presence of peptidase inhibitors. The MVD and GPI bioassays (31) were also run to confirm the activity of ligands **1–9**. The IC_{50} values were determined by standard statistical methods (32, 34). Data for **10** (DPDPE) were determined previously (27, 30).

β-D-glucoside moiety, were chosen for further study to see whether analgesia (peptide transport) could be demonstrated in live mice.

Analgesia *in Vivo*. The glycosylated peptides **4** and **5** were administered intraperitoneally (i.p.) to mice and antinociception was determined with two standard bioassays, the warm-water tail-flick test (Fig. 2*A*) and the hot-plate test (Fig. 2*B*). These tests measure the amount of time required for mice to react to standardized noxious stimuli. Substances which increase the reaction time are said to display antinociceptive effects, which may be interpreted as a measure of analgesia. Both glycopeptides **4** and **5** produced a significant and long-lasting analgesia in both tests, with peak effects seen ≈60 min after i.p. administration; this time course of analgesia is consistent with entry into the central nervous system.

Interestingly, the glycopeptides were more active in the hot-plate experiments than in the tail-flick experiments (60°C experiments; data not shown). Since the hot-plate response has been interpreted to require the activation of supraspinal mechanisms to inhibit a behavioral response, whereas the tail-flick procedure emphasizes blockade of a spinal reflex (39), this finding is suggestive of predominant activity of these glycopeptides at supraspinal sites. Such an interpretation is supported by further experiments with naloxone, an opioid antagonist which reverses the effects of opioid drugs. The

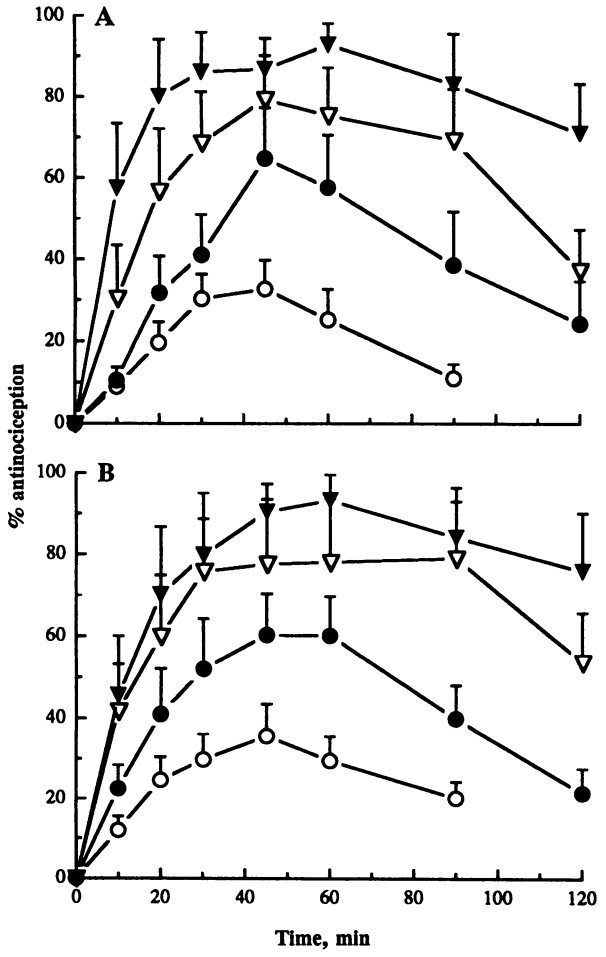


FIG. 2. The i.p. administration of glycopeptide **5** (Table 1) to mice produces analgesia. The i.p. administration of glycopeptide **5** (29) to mice led to dose-related antinociception in the 55°C warm-water tail-flick test (*A*) and in the 55°C hot-plate test (*B*). Glycopeptide **5** (Table 1) (LSZ1025) doses, i.p.: 30 mg/kg (○), 45 mg/kg (●), 60 mg/kg (▽), and 75 mg/kg (▼). The highest dose of glycopeptide **5** tested (75 mg/kg) produced long-lasting and maximal antinociception in both tests (26).

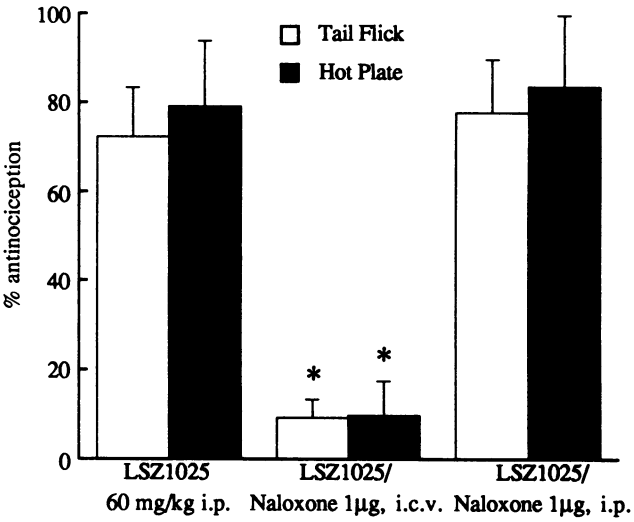


FIG. 3. Central administration of naloxone blocks analgesia. The effects of the opioid antagonist naloxone on antinociception were assessed with glycopeptide **5** (29) (60 mg/kg, i.p.) in the 55°C tail-flick (open bars) and 55°C hot-plate (filled bars) tests. Central (i.c.v.) administration (35) of a small dose (1 μg per mouse) of naloxone blocked antinociception activity of **5** in both tests (*, $P < 0.05$) (26). In contrast, the same total dose of naloxone given i.p. had no effect on antinociception. LSZ1025, glycopeptide **5** (Table 1).

cyclic glycopeptide **5** was given i.p. to mice, and antinociception was determined after 30 min in both the hot-plate and tail-flick assays. Administration of naloxone (1 μg per mouse) directly into the lateral cerebral ventricle (i.c.v.) 15 min after the glycopeptide produced a significant antagonism of antinociception (Fig. 3). As a control, the same dose of naloxone was given i.p. 15 min after the i.p. administration of **5**, to test the possibility that all of the naloxone gained entry into the blood circulation. In this case, i.p. naloxone given at 1 μg per mouse (the same dose which was effective as an antagonist when given i.c.v.) failed to alter the observed antinociceptive actions of **5**. These findings again support a site of action within the brain for glycopeptide **5**. As a further control, the unglycosylated peptides **8** and **9** were tested for analgesic activity in an identical fashion. No significant antinociceptive effects were detected for either **8** or **9** (data not shown).

DISCUSSION

The initial concept in this study was to utilize the mammalian facilitative glucose transporter or related carbohydrate transporters (40, 41) to deliver "natural" peptide messages across the BBB and other physiological barriers. Although other carbohydrates are enzymatically transported across the BBB, β-D-glucose seemed to be the logical choice because of the large influx of this sugar into the brain. About 20% of the glucose in the bloodstream is metabolized by the brain and all of it is actively transported across the BBB. It is possible that GLUT-1-mediated transport of our glycopeptide is responsible for the analgesic effects which have been observed.

The work with the enkephalin galactosides and glucosides done earlier by Rodriguez *et al.* (21) is consistent with GLUT-1-mediated transport. They observed that galactosyl enkephalin analogues were 1700 times more potent than glucosylated derivatives when the drugs were administered i.c.v. They attributed this effect to "diffusion" of the glucosyl peptide. Our suggestion is that diffusion of this substrate may be mediated by GLUT-1, which would not transport the galactoside out of the brain. Indeed, i.c.v. administration of glycopeptide **5** shows a very short duration of action, a result which is also consistent with transporter-mediated diffusion

out of the brain. We cannot rule out the possibility that other carbohydrate-mediated transport phenomena are involved—glycolipid transfer protein-mediated endocytosis, for example (13, 42). It is conceivable that the β-D-glucosides undergo enzymatic transformations either prior to or after BBB transport, although the stability studies (serum, $t_{1/2} \approx 80$ min; brain, $t_{1/2} > 7$ hr) do not support this idea.

The placement of the glycoside along the peptide is important for central nervous system activity. The glucoside-bearing peptides **4** and **5** were bound at both the μ- and δ-opioid receptors and showed considerable activity *in vivo* which lasted for at least 2 hr in the tail-flick and the hot-plate tests. Glycopeptides **1** and **2** were inactive, showing very poor binding at both the μ and the δ receptors. In this respect they may be similar to the morphine-3-glucuronate **B**, which is also inactive, even though it has been shown to cross the BBB.

Paradoxically, attachment of the β-D-glucose moiety actually decreases the lipophilicity of these peptides, while at the same time apparently increasing their transfer across a lipophilic barrier. The use of the body's own active-transport system to cross the BBB permits the utilization of highly water-soluble compounds which are easily administered without the use of organic solvents such as dimethyl sulfoxide. In fact, the β-D-glucosylated peptides were much more water soluble than their unglycosylated peptide counterparts. The glycopeptides used in this study show the expected pharmacodynamics of a typical central nervous system drug, with the analgesia slowly increasing to a maximum after 60 min and then declining over time. In addition, the effects of glycopeptide **5** were reversed by naloxone, a well-known opioid antagonist.

Conclusions. We have demonstrated that β-D-glucosylated opioid agonist peptides produce significant analgesia when administered peripherally. Furthermore, the observed analgesia can be attributed to central effects, which implies penetration of the BBB. The phenomena observed in this study are even more remarkable when one considers that the peptides must also migrate from the i.p. space into the bloodstream before entering the brain capillaries. This migration is facilitated because the glycopeptides are very water soluble. This bodes well for the exciting prospect of producing analgesia with limited addiction and tolerance and for the treatment of many disease states with the brain's own peptide messengers. The enkephalin analogues were not only metabolically stable for extended periods of time *in vivo* but able to cross the BBB to produce prolonged analgesia. Thus, the use of a carbohydrate recognition element to effect transport across the BBB significantly expands the utilization of the brain's own messengers and carbohydrate transport system to influence brain function.

We thank the National Institute on Drug Abuse (DA06284), the National Science Foundation (CHE-9201112), and the Arizona Disease Control Research Commission for support of this work.

1. Hruby, V. J., Kazmierski, W., Kawasaki, A. M. & Matsunaga, T. O. (1991) in *Peptide Pharmaceuticals*, ed. Ward, D. J. (Open Univ. Press, Buckingham, U.K.), Chapt. 5, pp. 135–184.
2. Knapp, R. J., Davis, T. P., Burks, T. F. & Yamamura, H. I. (1991) in *Peptide Pharmaceuticals*, ed. Ward, D. J. (Open Univ. Press, Buckingham, U.K.), Chapt. 7, pp. 210–242.
3. Pardridge, W. M. (1991) *Peptide Drug Delivery to the Brain* (Raven, New York).
4. Ermisch, A., Brust, P., Kretzschmar, R. & Rühle, H. (1993) *Physiol. Rev.* **73**, 489–527.
5. Banks, W. A. & Kastin, A. J. (1990) *Am. J. Physiol.* **259**, E1–E10.
6. Plattner, J. J. & Norbeck, D. W. (1989) in *Drug Discovery Technologies*, eds. Clark, C. R. & Moos, W. R. (Halsted, Chichester, U.K.), pp. 92–128.
7. Bickel, U., Yoshiwawa, T., Landaw, E. M., Faull, K. F. & Pardridge, W. M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2618–2622.
8. Bungaard, H. (1992) *Adv. Drug Delivery Rev.* **8**, 1–38.
9. Bodor, N., Prokai, L., Wu, W.-M., Farg, H., Jonalagadda, S., Kawamura, M. & Simpkins, J. (1992) *Science* **257**, 1698–1700.
10. Banks, W. A., Kastin, A. J. & Ehrensing, C. A. (1993) *J. Neurosci. Res.* **35**, 690–695.
11. Frank, H. J. L., Pardridge, W. M., Morris, W. L., Rosenfeld, R. G. & Choi, T. B. (1986) *Diabetes* **35**, 654–661.
12. Fishman, J. B., Rubin, J. B., Handrahan, J. V., Conner, J. R. & Fine, R. E. (1987) *J. Neurosci. Res.* **18**, 299–304.
13. Sasaki, T. & Abe, A. (1989) *Methods Enzymol.* **179**, 559–566.
14. Rakíc, L., Begley, D. J., Davson, H. & Zlokovic, B. V., eds. (1988) *Peptide and Amino Acid Transport Mechanisms in the Central Nervous System* (Macmillan, London).
15. Namane, A., Gouyette, C., Fillion, M. & Huynh-Dinh, T. (1992) *J. Med. Chem.* **35**, 3039–3044.
16. Bell, G. I., Burant, C. F., Takeda, J. & Gould, G. W. (1993) *J. Biol. Chem.* **268**, 19161–19164.
17. Fisher, J. F., Harrison, A. W., Bundy, G. L., Wilkinson, K. F., Rush, B. D. & Ruwart, M. J. (1991) *J. Med. Chem.* **34**, 3140–3143.
18. Filira, F., Biondi, L., Cavaffion, F., Scolaro, B. & Rocchi, R. (1990) *Int. J. Pept. Protein Res.* **36**, 86–96.
19. Horvat, S., Horvat, J., Vargadefterdarvocic, L., Pavelic, K., Chung, N. N. & Schiller, P. W. (1993) *Int. J. Pept. Protein Res.* **41**, 399–404.
20. Vargadefterdarvocic, L., Horvat, S., Chung, N. N. & Schiller, P. W. (1992) *Int. J. Pept. Protein Res.* **39**, 12–17.
21. Rodriguez, R. E., Rodriguez, F. D., Sacristàn, M. P., Torres, J. L., Valencia, G. & Garcia Antòn, J. M. (1989) *Neurosci. Lett.* **101**, 89–94.
22. Mulder, G. J. (1992) *Trends Pharmacol. Sci.* **13**, 302–304.
23. Osborne, R., Joel, S., Trew, D. & Slevin, M. (1988) *Lancet* **i**, 828.
24. Rapaka, R. S. & Porreca, F. (1991) *Pharm. Res.* **8**, 1–8.
25. Hawkins, K. N., Knapp, R. J., Lui, G. K., Guyla, K., Kazmierski, W., Wan, Y.-P., Pelton, J. T., Hruby, V. J. & Yamamura, H. I. (1989) *J. Pharmacol. Exp. Ther.* **248**, 73–80.
26. Porreca, F., Mosberg, H. I., Hurst, R., Hruby, V. J. & Burks, T. F. (1984) *J. Pharmacol. Exp. Ther.* **230**, 341–348.
27. Hruby, V. J., Toth, G., Gehrig, C. A., Kao, L.-F., Knapp, R., Liu, G. K., Yamamura, H. I., Kramer, T. H., Davis, P. & Burks, T. F. (1991) *J. Med. Chem.* **34**, 1823–1830.
28. Weber, S. J., Greene, D. L., Sharma, D. D., Yamamura, H. I., Kramer, T. H., Burks, T. F., Hruby, V. J., Hersh, L. B. & Davis, T. P. (1991) *J. Pharmacol. Exp. Ther.* **259**, 1109–1117.
29. Polt, R., Szabò, L. Z., Treiberg, J. T., Li, Y. & Hruby, V. J. (1992) *J. Am. Chem. Soc.* **114**, 10249–10258.
30. Mosberg, H. I., Hurst, R., Hruby, V. J., Gee, K., Yamamura, H. I., Gallegan, J. J. & Burks, T. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5871–5874.
31. Shook, J. E., Pelton, J. T., Wire, W. S., Hirning, L. D., Hruby, V. J. & Burks, T. F. (1987) *J. Pharmacol. Exp. Ther.* **240**, 772–777.
32. Statistical Consultants (1986) *Am. Stat.* **40**, 52–60.
33. Vaughn, L. K., Knapp, R. J., Toth, G., Wan, Y.-P., Hruby, V. J. & Yamamura, H. I. (1989) *Life Sci.* **45**, 1001–1008.
34. Munson, P. J. & Robard, D. (1980) *Anal. Biochem.* **107**, 220–239.
35. Haley, T. J. & McCormick, W. G. (1957) *Br. J. Pharm. Chemother.* **12**, 12–15.
36. Tallarida, R. J. & Murray, R. B. (1987) *Manual of Pharmacological Calculations with Computer Programs* (Springer, New York), 2nd Ed.
37. Dyson, H. J., Rance, M., Houghten, R. A., Lerner, R. A. & Wright, P. E. (1988) *J. Mol. Biol.* **201**, 161–200.
38. Hruby, V. J. & Gehrig, C. C. (1989) *Med. Res. Rev.* **9**, 343–401.
39. Yaksh, T. L. & Rudy, T. A. (1978) *Pain* **4**, 299–359.
40. Kaback, H. R. (1986) *Annu. Rev. Biophys. Biophys. Chem.* **15**, 270–319.
41. Wright, E. M. (1993) *Annu. Rev. Physiol.* **55**, 575–589.
42. Anderson, R. G. W., Kamen, B. A., Rothberg, K. G. & Lacey, S. W. (1992) *Science* **255**, 410–411.