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Glycodermorphins: opioid peptides with potent and prolonged analgesic activity and enhanced blood-brain barrier penetration

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1 In order to improve the *in vivo* stability of the opioid peptide dermorphin we synthesized O-βglucosylated analogs ([Ser^7-O-βGlc]dermorphin and [Ser^7-O-βGlc(Ac)$_4$]-dermorphin) and C-αgalactosylated analogs ([Ala^7-C-αGal]dermorphin and [Ala^7-C-αGal(Ac)$_4$]-dermorphin).

2 O- and C-glycosylation of dermorphin halved the peptide affinity for brain μ-opioid receptors and the biological potency in guinea-pig ileum assay (GPI). Despite their lower opioid receptor affinity, when administered intracerebroventricularly (i.c.v., 8–40 pmol) and subcutaneously (s.c., 0.5–3 μmol kg^{-1}) in rats, glycosylated analogs were two times more potent than dermorphin in reducing the nociceptive response to radiant heat. Acetylation of sugar hydroxyl groups reduces 5–10 times both biological activity on GPI and μ-receptor affinity, whereas the antinociceptive potency was equal to (i.c.v.) or only two-three times lower (s.c.) than dermorphin potency.

3 Blood-Brain Barrier Permeability Index (BBB-PI) of the glycodermorphins was significantly higher than that of dermorphin, indicating a facilitated entry into the brain: O-β-linked glucoconiugates are expected to enter CNS by the glucose transporter GLUT-1 of the endothelial barrier. However the calculated BBB-PI for the C-αgalactoside was about two times higher than that of the O-βglucoside, excluding the implication of GLUT-1 that is known to be selective for O-β-links and preferring for the exose glucose.

4 The enhanced brain permeability with the subsequent decrease in peripheral dosage of these opioid peptides did not result in lowering constipation.

Keywords: peptide glycosylation; enzyme degradation; CNS permeability; specialized transport systems

Introduction

Analgesia produced by opioids is thought to be a centrally mediated event. Therefore only those agents which are able to cross the blood-brain barrier (BBB) and the blood-cerebral spinal fluid (CSF) barrier are likely to produce analgesia after peripheral administration. The capacity of any particular molecule to penetrate the BBB depends essentially on its free diffusion across the endothelial cell membranes, i.e. on its lipophilicity, or on its specific affinity for a carrier or a receptor mediated transport system. It was initially assumed that highly polar peptides could not enter into the central nervous system (CNS) *via* the BBB (Pardridge & Mietus, 1981) and that the eventual entry of some peptides is correlated only with their lipophilicity. Now it is generally accepted that different categories of peptides can enter or exit the CNS through the endothelial cell membranes of brain microvessels at a rate higher than that accounted by passive diffusion. Williams *et al.* (1996) evidenced that DPDPE enters the CNS of anesthetized rats by a saturable mechanism found at the BBB, with the blood-CSF barrier playing only a minor role in the brain uptake of the peptide. Banks and Kastin (1994) suggested that at least four different transport systems, named PTS-1, -2, -3, and -4, can accelerate the entrance of opioids and opioid-related peptides into the brain. In a previous work we demonstrated that a transport system selective for deltorphins accelerates the entrance of opioid peptides in the bovine brain capillaries (Fiori *et al.*, 1997). Nevertheless, the passage of peptides across the BBB is extremely limited in comparison with more diffusible compounds, and several strategies have been used to enhance peptide delivery to the CNS. These strategies include increasing the peptide lipophilicity or cationic character for a better membrane diffusion or absorptive transport as well as the development of chimeric compounds with a peptide message covalently linked to transport vectors (Pardridge, 1991). Polt *et al.* (1994) reported that peripheral administration of the L-serinyl-β-D-glucoside analogue of [Met^5]enkephalin, i.e. [(β-D-Glc)Ser^2,Met^5]enkephalin, produces antinociception in mice and suggested that glucopeptides could enter CNS being transported through the endothelial barrier by the glucose transporter GLUT-1. Moreover the lower susceptibility to proteolytic enzymes induced by glycosylation may also contribute to enhance the analgesic effect by increasing the peptide half-life and raising its level in the blood and CNS. Previous studies (Kessler *et al.*, 1992) carried out on C-glycosylated peptides showed that their conformational features parallel those of the corresponding O-glycosylated derivatives, and that they are resistant to proteolysis and unable to form hydrogen bonds. The C-linked, the N-linked and the α-O-linked sugar conjugates are not expected to promote transport since glucose transporter is specific for β-O-linked glucosides. Therefore these glycosylated peptides could represent useful tools to establish the role of enzymatic protection afforded by glycosylation in enhancing peptide CNS permeability. The μ-selective opioid peptide demorphin displays a longer half-life in comparison with enkephalins and is able to enter the CNS at a moderate rate (Negri & Improta, 1984). Dermorphin administration by intracerebroventricular (i.c.v.) and intrathecal (i.t.) route induces antinociception in nanomolar dose range, but micromolar doses are needed to obtain comparable effect by parentheral administration. We have already demonstrated that only residues 1–4 of the dermorphin sequence participate

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in the μ-receptor binding and opioid activity (Melchiorri *et al*., 1991). We thus prepared some dermorphin analogs bearing O- and C-linked glycosyl residues on the C-terminal amino acid residue and used these glycoconjugates to assess if glucose transporters could be responsible for the enhancement of the brain penetration. A second target was to produce stable opioid peptide analogues which, by peripheral administration, could act as potent, long lasting analgesics with enhanced brain permeability. Theoretically, high brain permeability is a prerequisite for reducing the peripheral analgesic dose and hence attenuating peripheral opioid side effects as delayed gastric emptying, constipation, contraction of the sphincter of Oddi and inhibition of urinary voiding reflex. The β-O-glucosylated and the α-C-galactosylated dermorphin analogues and their acetylated derivatives, in which the sugar hydroxyl groups are not available for sugar transporters, were thus compared for their capacity to induce antinociception in rats after central and peripheral administration and to reduce the colonic propulsion after peripheral administration.

Methods

Peptides synthesis

The following glycopeptides were prepared by the solid phase procedure: (**I**) H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-[βGlc(Ac)$_4$]Ser-NH_2 [Ser^7-O-βGlc(Ac)$_4$]dermorphin; (**II**) H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-(βGlc)Ser-NH_2 [Ser^7-O-βGlc]dermorphin; (**III**) H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-[αGal(Ac)$_4$]Ala-NH_2 [Ala^7-C-αGal(Ac)$_4$]dermorphin; (**IV**) H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-(αGal)Ala-NH_2 [Ala^7-C-αGal]dermorphin; Fmoc-[O-βGlc (Ac)$_4$]Ser-OH (Filira *et al*., 1990) and Fmoc-[C-αGal(AC)$_4$] Ala-OH, (Kessler *et al*., 1992) were synthesized according to the literature. Assemblies of the glycosylated tetra-acetylated analogues **I** and **III** on the Applied Biosystem mod 431 A Peptide Synthesizer were performed by the standard procedure starting with Rink Amide MBHA resin [4(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamido-norleucyl-4-methyl-benzhydrylamine polystyrene] (Novabiochem). The Fmoc strategy and a single coupling protocol with DCC/HOBT in NMP was used through all syntheses. Coupling yields were higher than 99.6%. To prevent the possible intrachain aminolysis at the dipeptide stage (Pedrose *et al*., 1986) and the consequent peptide chain loss in form of dichetopiperazine, the dipeptide Fmoc-Tyr(tBu)-Pro-OH containing the third and second residues was used in the acylation step. Final yields were in the range 58–62%. Deacetylation was achieved by adding excess hydrazine hydrate to a methanolic solution of **I** and **III**. Average deacetylation yield was 90%. Tetracetylated (**I, III**) and deacetylated (**II, IV**) glycopeptide analogues were characterized by reverse phase analytical HPLC [column Aquapore Octyl RP-300, 222×4.6 mm, Brownlee Lab. Load 10 μg, flow rate 1.5 ml : min. Eluant A (0.1% TFA in 90% aqueous CH_3CN) and B (aqueous 0.1% TFA) were used for preparing binary gradients].

Opioid receptor binding

Binding of the peptides to δ- and μ- opioid receptors was assayed on crude membrane preparations from adult male rat brain (Wistar, 250–300 g) as previously described (Melchiorri *et al*., 1991). The δ-binding sites were selectively labeled with [^{3}H][D-Ala^2, Asp^4]deltorphin (0.3 nM), the μ-sites with [^{3}H][D-Ala^2, MePhe^4, Glyol^5]enkephalin ([^{3}H]DAMGO, 0.5 nM). Competition curves were determined in triplicate. The inhibition constant (K_i) of the various peptides was calculated from competitive binding curves with the computer program, Ligand (Munson & Rodbard, 1980). Data obtained from four independent measurements are presented as the arithmetic mean $\pm$ s.e.mean.

Activity on isolated organ preparations

Preparations of the myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea pigs (GPI) and preparations of mouse vas deferens (MVD) were used for field stimulation with bipolar rectangular pulses of supramaximal voltage (Melchiorri *et al*., 1991). Agonists were evaluated for their inhibition of the electrically evoked twitch. The results were expressed as the IC_{50} values obtained from concentration-response curves (Tallarida & Murray, 1986). IC_{50} values represent the mean $\pm$ s.e.mean of not less than eight tissue samples.

Test of peptide degradation

Peptides were incubated with rat brain homogenates or with rat liver homogenates in 50 mM Tris-HCl, pH 7.4, at 37°C for 0–60 min (200 nmol of peptide per 100 mg of tissue, ml^{-1}). At the end of each incubation (0, 5, 10, 20, 40 and 60 min), an equal volume of methanol was added to each sample to precipitate proteins and stop enzymatic activity. Samples were then centrifuged at $13{,}000 \times g$ on a Beckman -TJ6 centrifuge for 15 min. The supernatant was collected and the recovered biological activity was evaluated by pharmacological assays on GPI preparations, and expressed as percent of the recovery at time 0 (control). The results are mean $\pm$ s.e.mean of three experiments.

Animals

Male Wistar rats (220–280 g) were used for the experiments. They were housed at 22 ± 2°C, with food and water *ad libitum*. A standard light/dark cycle was maintained with a time-regulated light period from 06 h to 18 h. The IASP guidelines on ethical standards for investigations of experimental pain in animals were followed.

Subcutaneous (s.c.) injections Compounds were dissolved in sterile saline and administered to the rats in a volume of 2 ml kg^{-1} of body weight. Control rats received saline at 2 ml kg^{-1} dose. Each animal received one injection only.

Intracerebroventricular (i.c.v.) injections Under ketamine-xylazine anesthesia (60 mg kg^{-1} + 10 mg kg^{-1}, i.p.) a plastic guide cannula (Linca, Tel-Aviv, Israel), was stereotaxically implanted over the left lateral ventricle of the rat brain (AP = −1 mm, L = +1.8 mm relative to the bregma, according to data from Paxinos and Watson, 1982) as previously described (Negri *et al*., 1995). After one week-recovery from surgery, drugs or control solutions were i.c.v. injected in a constant volume of 5 μl in awake rats. Each rat received one injection only. At the end of the experiments, the proper position of the i.c.v. injections was ascertained by inspection of the brain ventricles after an injection of methylene blue.

Tests of antinociception

Every dose of each peptide was evaluated in groups of 6–8 animals. Antinociception was assessed by exposing the rat tail

to radiant heat (D'Amour & Smith, 1941) and the latency to a rapid tail-flick was recorded with the baseline cutoff and the maximal possible latencies set at 4 s and 12 s, respectively. The degree of analgesia was expressed as percentage maximum possible effect (% MPE) according to the equation: % MPE = [(test latency − control latency)/(12 − control latency)] × 100. The tail-flick latency was measured before drug treatment (control) and every 15 min after drug injections, during the first hour, and every 30 min thereafter, until analgesia disappeared. A computer program (Prisma, GraphPad, CA, U.S.A.) was used to calculate the mean peak effect and the mean area under the time-response curve (AUC) for each dose. Doses that produced peak effects between 20% and 80% MPE were plotted into a log dose-response curve and AD_{50} and AD_{80} values were calculated as doses that produced 50% and 80% MPE, respectively (Tallarida & Murray, 1986).

Blood brain barrier permeability index (BBB-PI)

Since in addition to BBB permeability, differences in degradation and/or elimination rates affect peptide availability at the central sites of action, we multiplied each dose for the ratio of peak effect to AUC to obtain normalized dose values. The ratio of i.c.v. normalized AD_{80} to s.c. normalized AD_{80} provided an indirect measure of the CNS permeability of the peptide defined as BBB-PI (Fiori *et al*., 1997).

Colonic propulsion

Colonic propulsion was measured according to bead expulsion method of Raffa & Jacoby (1990). Fifteen minutes after s.c. administration of saline (control) or peptides, at doses ranging from AD_{10} to AD_{50}, a single 5 mm glass bead was inserted into the distal colon of rats. The time required for expulsion of the glass bead was determined for each animal. Inhibition of colonic propulsion was measured as the increase in mean expulsion time (MET) of the glass bead and expressed as the percent of maximum possible effect (MPE) according to the formula: $MPE = [(MET_p - MET_s)/(120 - MET_s)] \times 100$, where MET_p is the mean expulsion time in peptide treated animals (eight animals per group), MET_s is that in saline-treated animal (eight animals) and 120 is the cut-off time.

Drugs

For *in vivo* studies all the peptides and naloxone (S.A.L.A.R.S. Como, Italy) were dissolved in normal saline. The μ-binding site was selectively labeled with 0.5 nM [^{3}H]DAMGO ([^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, 55.3 Ci/mmol, Amersham, UK); the δ-binding sites with 0.3 nM [^{3}H]DELT I ((3,5-^{3}H-tyrosyl)-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; 47 Ci/mmol, custom synthesized by CRB, Cambridge, U.K.).

Results

Receptor binding and GPI-MVD assay

In the dermorphin sequence, glucosylation of the hydroxyl side chain function of C-terminal serine halved the μ-opioid receptor affinity and the biological activity on GPI and MVD test. Acetylation of the four hydroxyl functions of glucose strongly reduced (7 times) the affinity of glucodermorphin for μ-opioid receptors and further attenuated the potency on GPI but not MVD test. Substitution of Ser^7 with Ala in the dermorphin sequence did not induce any change both in opioid receptor affinity and in GPI-MVD biological potency. αGalactoside of [Ala^7]dermorphin and its acetylated derivative displayed a decrease in the opioid receptor affinity and biological activity similar to that observed with glucosylated dermorphine (Table 1). The affinity for δ-opioid receptors was unaffected by O-βglucosylation, whereas it was significantly reduced by C-αgalactosylation of the dermorphin molecule.

Peptide degradation

The decay of biological activity of [Ser^7-O-βGlc]dermorphin ($t_{1/2}$ = 45 min) in rat brain homogenates was slightly slower than that of dermorphin ($t_{1/2}$ = 30 min), but in liver homogenates it was significantly reduced ($t_{1/2}$ = 38 *vs* 15 min) in comparison with the dermorphin decay (Figure 1a, b). Thus O-β glucosylation of the Ser^7 hydroxyl group afforded protection against enzymatic breakdown. Such a protection is more evident in liver than in brain homogenates because the rate of degradation of the dermorphin molecule is faster in liver than in brain homogenates. Acetylation of the four hydroxyl groups produced an unusual pattern of enzymatic degradation of [Ser^7-O-βGlc(Ac$_4$)]dermorphin (Figure 1). From ten to twenty minutes after addition of the peptide to brain homogenates, the bioactivity of the incubation medium extract increased, instead of decreasing, demonstrating that peptides more potent than the parent compound were produced (Figure 1a). [β-Glc(Ac)$_4$]-dermorphin may function as a prodrug with its degradation products, dermorphin, (1-6)dermorphin and (1-5)dermorphin, being responsible for the early increase in biological activity. As shown in Table 1, dermorphin and its

Table 1 μ and δ opioid receptor affinities and biological activities on guinea-pig ileum (GPI) and mouse vas deferens (MVD) of dermorphin analogues and homologues

	K_i, nM		*IC_{50}, nM*	
Peptide	*μ [^{3}H]DAGO*	*δ [^{3}H]DELT-I*	*GPI*	*MVD*
Dermorphin	1.1 ± 0.2	929 ± 41	1.5 ± 0.2	19 ± 1.8
[Ser^7-O-βGlc]dermorphin	2.4 ± 0.3	1230 ± 150	3.5 ± 0.41	40 ± 5
[Ser^7-O-βGlc(Ac)$_4$]dermorphin	7.9 ± 0.9	1400 ± 147	8.2 ± 0.7	18.1 ± 2.9
[Ala^7]dermorphin	1.2 ± 0.2	1385 ± 139	1.6 ± 0.2	24 ± 2
[Ala^7-C-αGal]dermorphin	2.5 ± 0.4	2500 ± 190	4.0 ± 0.5	55 ± 4.5
[Ala^7-C-αGal(Ac)$_4$]dermorphin	11.2 ± 2.1	> 5000	6.0 ± 0.5	65 ± 7.1
(1-6)dermorphin*	1.6 ± 0.11	320 ± 19	2.73 ± 0.35	43.2 ± 3.9
(1-5)dermorphin*	0.98 ± 0.11	88 ± 8.6	2.88 ± 0.37	23.8 ± 3.6

K_i, equilibrium dissociation constant of the competing ligand; IC_{50}, agonist concentration that produced 50% inhibition of the electrically evoked twitch; *data from Melchiorri *et al*., *Eur. J. Pharmacol.* **195,** 201 – 207, 1991.

shorter homologues display μ-opioid receptor affinity and *in vitro* biological potency from three to four times higher than that of [β-Glc(Ac)$_4$]dermorphin. This peptide was also significantly more resistant to liver enzymes (Figure 1b).

Antinociception

In the rat tail-flick test, i.c.v. and s.c. administration of dermorphin and its glycosylated derivatives elicited a dose-dependent antinociception. By i.c.v. administration the AD_{50} values of both O- and C-tetra-acetyl-glycosylated dermorphin analogues (36.3[31.7, 44.1] and 32.1[24.4, 44.1] pmol per rat, respectively) did not significantly differ from that of dermorphin (30[24,38] pmol per rat), but O-glucosylated and C-galactosylated analogs were about two times more potent (13.5[11.2, 16]; 22.6[21.5, 23,8] pmol per rat, respectively) than dermorphin (Figure 2a). Doses of 22 pmol [Ser^7-O-βGlc]dermorphin and 30 pmol [Ala7-C-αGal]dermorphin produced antinociceptive responses that peaked (MPE = 80%) between 45 and 60 min and were equivalent to 60 pmol of dermorphin (Figure 3a). As expected from degradation experiments, acetylation of glycodermorphins prolonged the antinociceptive response. Equianalgesic doses of the acetylated analogs (50 pmol of [Ser^7-O-βGlc(Ac)$_4$]dermorphin and 60 pmol of [Ala^7-C-αGal(Ac)$_4$]dermorphin) peaked after 60 min but lasted twice as long as dermorphin (Figure 3a).

By s.c. administration dose-response curve of dermorphin displayed a significantly slower slope than the dose-response curves of the glycosylated peptides. Therefore, AD_{50} of [Ala^7-C-αGal]dermorphin (0.64[0.52, 0.8] μmol kg^{-1}) was 2.3 times lower than dermorphin AD_{50} (1.5[1.3, 1.6] μmol kg^{-1}) but AD_{80} of the galactosyl derivative was 3.5 times lower than AD_{80} of the parent peptide. For [Ser^7-O-βGlc]dermorphin the AD_{50} (0.8[0.5, 1.1] μmol kg^{-1}) and AD_{80} values were 1.9 and 2.5 times lower than the corresponding values of dermorphin, respectively. The AD_{50} values of acetyl-glycosylated analogs (3.7[3.0,4.5] and 5.3[4.0,7.0] μmol kg^{-1} respectively for [Ser^7-O-βGlc(Ac)$_4$]dermorphin and [Ala^7-C-αGal(Ac)$_4$]dermorphin)

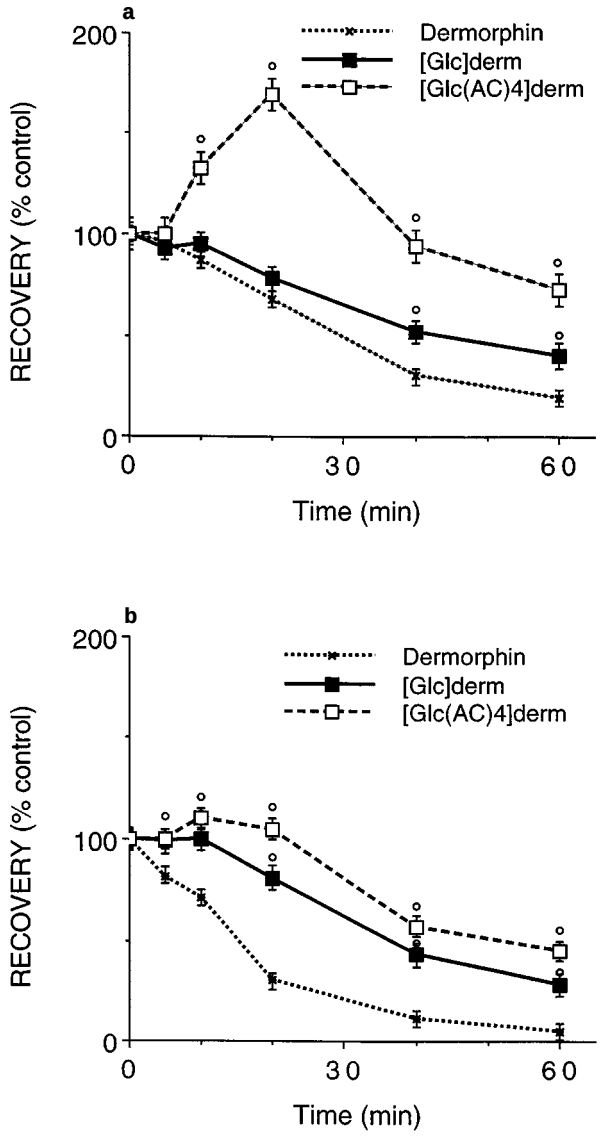

Figure 1 Time course of peptide degradation by rat brain (a) and liver (b) homogenates. Each point represents the mean ± s.e.mean of three experiments. °$P<0.05$ *vs* dermorphin. [Glc]derm: [Ser^7-O-βGlc]dermorphin. [Glc(AC)4]derm: [Ser^7-O-βGlc(Ac)$_4$]dermorphin.

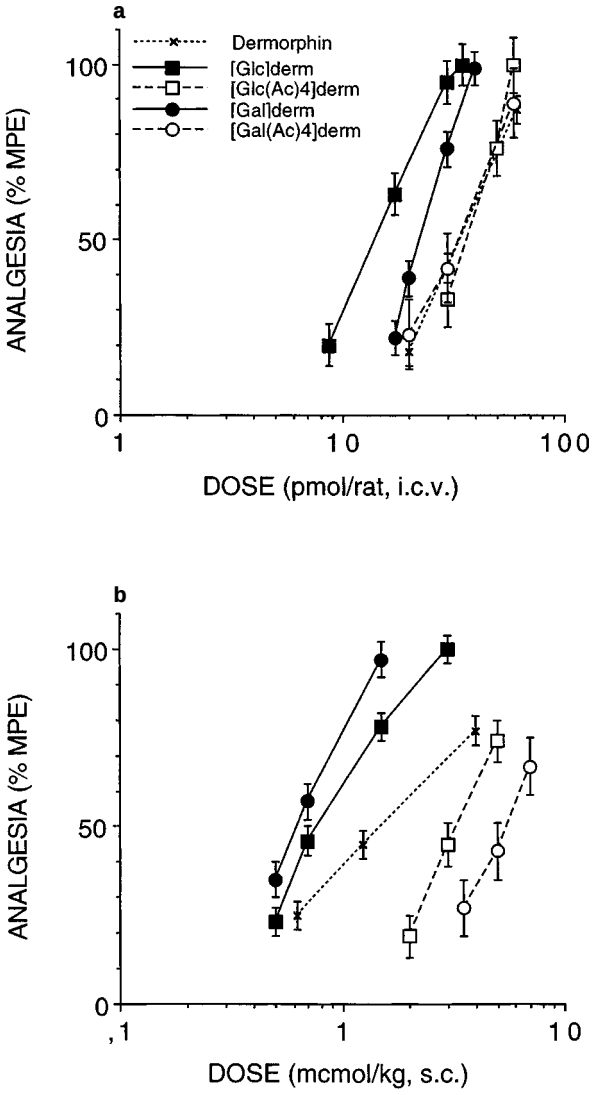

Figure 2 Dose-response curves of the antinociception produced by dermorphin and by the four glycosylated analogues of dermorphin in rats: (a) i.c.v. administration; (b) s.c. administration. Each point represents the mean antinociceptive peak effect of 8 rats ± s.e.mean.

were 2.5–4 times higher than dermorphin AD_{50}, but the AD_{80} values were only 1.3–2 times higher than dermorphin AD_{80} (Figure 2b). Antinociception induced by s.c. acetylated glycodermorphins lasted considerably longer than that induced by equianalgesic doses of the corresponding deacetylated glycosides (Figure 3b).

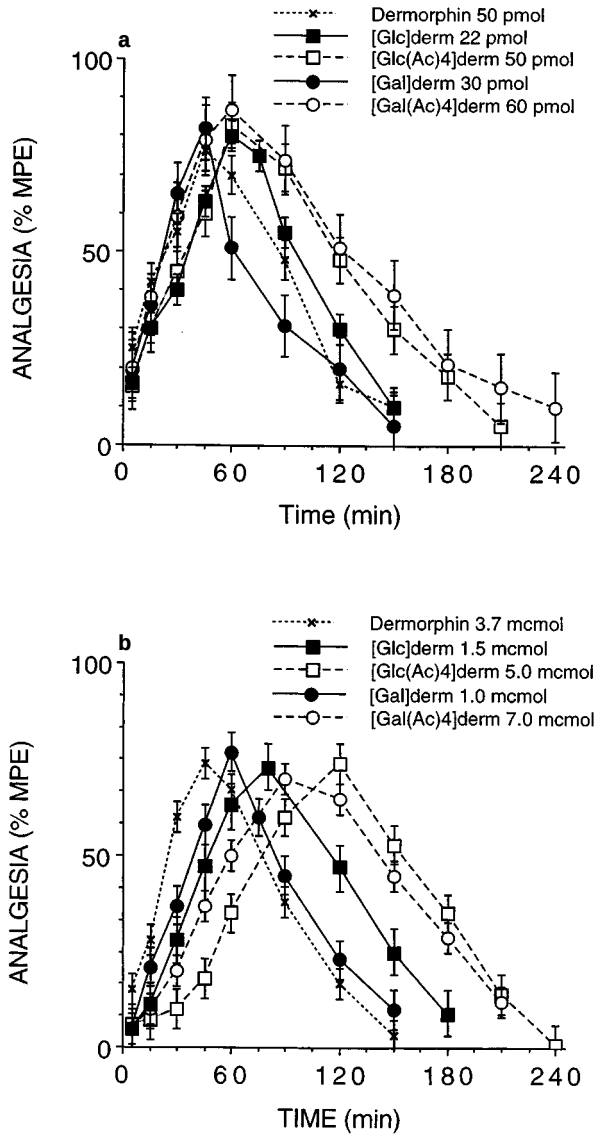

Figure 3 Time course of the antinociception produced by equianalgesic doses of dermorphin and of the four glycosylated analogs of dermorphin. (a) i.c.v. doses are pmoles per rat. (b) s.c. doses are μmoles kg^{-1}. Each points represents the mean antinociceptive effect of 8 rats $\pm$ s.e.mean.

Naloxone antagonism and site of action

Antinociception produced by i.c.v. and s.c. administration of glycodermorphins and their acetylated analogs was antagonized by s.c. injection of low dose of naloxone (0.5 mg kg^{-1}, s.c., 15 min before peptide injection) (data not shown). To ascertain if the antinociception produced by s.c. administration of glycodermorphins was due to activation of brain opioid receptors, naloxone (1 μg/rat) was administered i.c.v. 15 min after the s.c. injection of 2 μmol kg^{-1} of [Ser^7-O-βGlc]dermorphin and the antinociceptive response obtained was compared with that evoked by s.c. injection of the same dose of the peptide followed, 15 min after, by an i.c.v. injection of 5 μl saline. I.c.v. naloxone produced an intense and significant decrease in the antinociceptive response produced by s.c. [Ser^7-O-βGlc]dermorphin. Because the same dose of naloxone given s.c. failed to alter the peptide antinociceptive effect, glucodermorphin-induced antinociception must be considered of central origin (Figure 4).

CNS permeability

Blood-Brain Barrier Permeability Index (BBB-PI) was calculated as the ratio of i.c.v. normalized AD_{80} to s.c. normalized AD_{80}. Thus, compounds that more easily enter the CNS display higher BBB-PI values (Table 2). [Ser^7-O-βGlc]dermorphin and [Ala^7-C-αGal]dermorphin display values 1.6 and 2.7 times higher than dermorphin indicating a

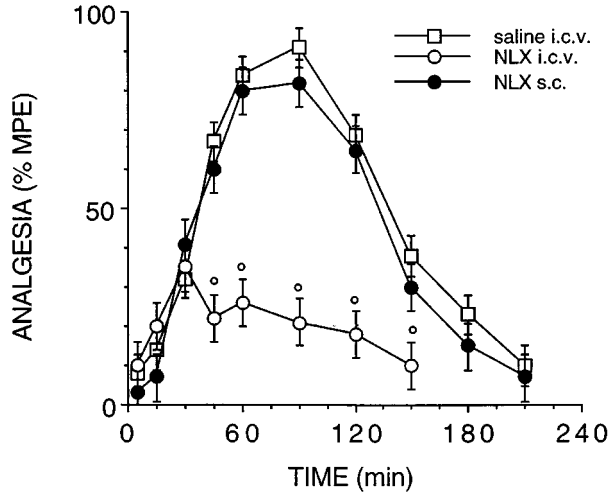

Figure 4 Antinociception induced by the s.c. administration of 2 μmol kg^{-1} of [Ser^7-O-βGlc]dermorphin together with: 5 μl/rat of saline i.c.v. (control, saline i.c.v.), 1 μg/rat of naloxone i.c.v. (NLX i.c.v.) or 1 μg/rat of naloxone s.c. (NLX s.c.). Each points represents the mean antinociceptive effect of 5 rats $\pm$ s.e.mean. $^{\circ}P<0.001$ *vs* saline i.c.v.

Table 2 Analgesia and relative CNS penetration of dermorphin analogues

	Analgesia (AD_{80})						*Normalized AD_{80} ratio*
	I.C.V.			*S.C.*			
Peptide	(nmol)	×	[MPE/AUC]	(nmol)	×	[MPE/AUC]	*I.C.V./S.C.*
Dermorphin	0.05	×	0.011	3700	×	0.013	1.14×10^{-5}
[Ser^7-O-βGlc]dermorphin	0.022	×	0.012	1500	×	0.009	1.95×10^{-5}
[Ser^7-O-βGlc(Ac)$_4$]dermorphin	0.05	×	0.0084	5000	×	0.0085	0.98×10^{-5}
[Ala^7-C-αGal]dermorphin	0.030	×	0.0147	1000	×	0.0132	3.34×10^{-5}
[Ala^7-C-αGal(Ac)$_4$]dermorphin	0.060	×	0.0079	7000	×	0.008	0.89×10^{-5}

facilitated entry into the brain, despite of the decreased lipophilicity induced by the glycoside moiety. [Ser^7-O-βGlc(Ac)$_4$]dermorphin and [Ala^7-C-αGal(Ac)$_4$]dermorphin show values 2 and 3.7 times lower than the corresponding non-acetylated analogs in spite of acetylation increased lipophilicity.

Colonic transit

The inhibitory effects on colonic propulsion induced by the two glycodermorphins in comparison with dermorphin were measured with bead expulsion test (Table 3), after s.c. injection. At equianalgesic doses the rank order of constipation potency was: [Ser^7-O-βGlc(Ac)$_4$]dermorphin > [Ser^7-O-βGlc]dermorphin > = dermorphin.

Discussion

Dermorphin has been shown to be an extremely potent analgesic when administered i.c.v. in rats, being 500–1000-fold more potent than morphine. However after peripheral administration, dermorphin is equipotent to morphine: only a small fraction (<0.0005%) of intravenously injected dermorphin enters the brain (Negri and Improta, 1984) whereas BBB-PI of morphine is 660-fold higher than that of dermorphin (Fiori *et al*., 1997). We have previously demonstrated that plasma half-life of dermorphin is 1.3 min, the peptide being rapidly destroyed in kidney and liver to di-tri- and tetra N-terminal peptides that are biologically inactive. The rate of dermorphin metabolism in brain and in plasma is respectively 20 and 40 times slower than in liver and kidneys, moreover the intact dermorphin can be eliminated more slowly than breakdown products (Negri & Improta, 1984). Thus, CNS permeability and bioavailability appear to be the limiting factors for the analgesic potency of dermorphin after peripheral administration. In present experiments, by i.c.v. administration, glycodermorphins were found to be 2–3 times more potent than dermorphin in inducing analgesia in spite of their μ-receptors affinity was one half that of dermorphin, and acetylglycodermorphins were as potent as dermorphin, in spite of their μ -opioid receptor affinity were 7–9 times lower. The degradation profiles observed in brain and liver homogenates suggest that glycosylation of the C-terminal amino acid in the dermorphin molecule strongly delays the decay of biological activity and, by means of this enzymatic protection, may rise the blood and CSF level of the peptide. Protection against the loss of biological activity is even more evident for acetylglycosides which behave as a pro-drug that releases more active peptides upon incubation with brain homogenates. As previously demonstrated (Melchiorri *et al*., 1991) and shown in Table 1, both dermorhin and its shorter homologues of display μ-opioid receptor affinity and *in vitro* biological potency from three to four times higher than that of [β-Glc(Ac)$_4$]dermorphin. Acetylated glycosides probably act as pro-drug also *in vivo*. Although in *in vitro* binding assay acetyl-glycodermorphins displayed an opioid receptor affinity ten times lower than dermorphin, when injected i.c.v. and s.c. in rats these peptides produce intense central antinociception comparable to that of dermorphin. Because i.c.v. injection of a peripherally inactive dose of naloxone antagonized antinociception induced by s.c. delivered glycodermorphins, the site of action of these glycopeptides must be centrally located.

Present data also demonstrate that glycosylation enhance CNS permeability of dermorphin. Because glycosides are highly polar compounds, we cannot ascribe this increased CNS permeability to enhanced lipophilicity of the peptide derivative. Moreover acetylation of hydroxyl groups of the sugar moiety, which increases lipophilicity, instead to favor the CNS entry of the peptides, decreased it. These results led us to argue the existence of some facilitated transport mechanism for these peptides at CNS-blood barrier, which requires free sugar hydroxyl groups. However, because galactosides show a CNS permeability about two times higher than that of glucosides and GLUT-1 transporter has selective preference for the β-O-link and a very low affinity for galactose, the facilitated passage of these peptides into the brain cannot be ascribed to glucose transporter. In a previous report (Negri *et al*., 1995), we demonstrated that another opioid peptide [Lys^7]dermorphin has a relative high blood to brain rate of influx, which cannot be attributed to the poor lipid solubility of the peptide. In a subsequent paper (Fiori *et al*., 1997) we demonstrated that *in vitro* isolated bovine brain microvessels can take up opioid peptides (deltorphins) through a saturable, non concentrative permeation system, which is apparently distinct from previously described systems involved in the transport of neutral aminoacids or enkephalins. (Audus *et al*., 1992; Banks & Kastin, 1990). Absorptive-mediated endocytosis has been indicated for a dynorphin-like peptide, E-2078 (Terasaki *et al*., 1989) and for cationized proteins (Pardridge *et al*., 1990; Triguero *et al*., 1989). Recently, Thomas *et al*. (1997), using primary endothelium culture from bovine brain microvessels, demonstrated that a cationic prodrug of deltorphin has similar permeability coefficient to the parent compound even though it has larger molecular weight and a significant lower lipophilicity. Thus large evidence exists for different peptide transport systems that facilitate the passage across the blood-brain barrier and show some degree of selectivity for the peptide structure. This kind of facilitated transport might offer a tool for the optimization of systemic pharmacokinetics and circumvention of the CNS-blood barrier. However present data indicate that the enhanced brain permeability with the consequent decrease in peripheral dosage of opioids did not result in lowering constipation. It might be possible that the effective peripheral dose still exceeds the threshold for this effect. Yet, these results agree with those previously obtained by us and other researchers showing that, at least in rats, opioid-induced delay in gastro-intestinal transit and bladder emptying are mainly centrally mediated effects (Broccardo *et al*., 1982; Manara & Bianchetti, 1985; De Luca & Coupar, 1996).

Table 3 Inhibitory effects on colonic propulsion induced by subcutaneous administration of equianalgesic doses of the two glucodermorphins and dermorphin

	Colonic bead expulsion (%MPE)	
Compounds	AD_{30}	AD_{50}
[Ser^7-O-βGlc]dermorphin	31 ± 5	78 ± 7
[Ser^7-O-βGlc(Ac)$_4$]dermorphin	93 ± 10	100
Dermorphin	38 ± 6	61 ± 8

AD_{30}: s.c. dose inducing 30% of the maximum possible analgesic effect: [Ser^7-O-βGlc]dermorphin = 0.5 μmol kg^{-1}; [Ser^7-O-βGlc(Ac)$_4$]dermorphin = 3.0 μmol kg^{-1}; dermorphin = 0.5 μmol kg^{-1}. AD_{50}: s.c. dose inducing 50% of the maximum possible analgesic effect: [Ser^7-O-βGlc]dermorphin = 0.7 μmol kg^{-1}; [Ser^7-O-βGlc(Ac)$_4$]dermorphin = 4.0 μmol kg^{-1}; dermorphin = 1.0 μmol kg^{-1}.

In conclusion, glycosylation proved to be an effective tool for producing stable opioid peptides that, when given by peripheral administration, could act as potent analgesics with enhanced brain permeability and prolonged action.

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