Dermorphin and Deltorphin Glycosylated Analogues: Synthesis and Antinociceptive Activity after Systemic Administration†

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In the present paper we describe the synthesis of some dermorphin and deltorphin analogues β-O- and α-C-glycosylated on the C-terminal amino acid residue and report their opioid receptor affinity and selectivity as well as their analgesic potency after subcutaneous injection in mice.

Introduction

There is a considerable interest in the development of peptides active by peripheral administration and retaining high receptor selectivity. Several strategies have been used to enhance peptide delivery to the central nervous system (CNS).[1] Polt and co-workers[2] demonstrated that glucosylated enkephalins enter the CNS, being transported through the endothelial barrier by the glucose transporter GLUT-1, and retain their biological activity. Tomatis and co-workers[3] demonstrated that Tyr[7]-glycosylated analogues of dermorphin and deltorphin retain high δ- or μ-opioid receptor selectivity and, as systemic antinociceptive agents, are more potent than the parent unglycosylated peptide counterparts. O-Glycosylation increases the peptide resistance against tissue peptidases and consequently increases the peptide half-life and raises its concentration in biological fluids. The resistance to proteolysis is further enhanced in the C-glycosylated peptide analogues whose conformational features are similar to those of the corresponding O-glycosylated derivatives and thus retain the receptor selectivity of the parent compounds.[4]

Dermorphin and deltorphin are opioid heptapeptides isolated from frog skin and are highly selective ligands at μ- and δ-opioid receptors, respectively.[5−7] As an antinociceptive agent, dermorphin is 40000-fold less potent after subcutaneous (sc) administration than after intracerebroventricular (icv) administration.[8] When injected into the brain of mice and rats, the highly selective δ-opioid ligands deltorphin I and deltorphin II produce analgesia,[9,10] locomotor stimulation,[11] and motivational rewarding[12] without development of physical dependence[13] or respiratory depression.[14] However the exploration of the therapeutic potential of these δ-opioid agents has been hampered by the lack of derivatives active on peripheral injection.

In the present paper we describe the synthesis of some dermorphin and deltorphin analogues β-O- and α-C-glycosylated on the C-terminal amino acid residue and report their opioid receptor affinity and selectivity as well as their analgesic potency after subcutaneous injection in mice. These glycosylated derivatives could represent useful tools to study the mechanism of blood−brain transport of bioactive peptides and the role of glycosylation in enhancing peptide stability in biological fluids and tissues.

Results and Discussion

Peptide Synthesis. The glycosylated opioid peptide analogues listed in Table 1 were prepared by standard solid-phase procedure (0.25-mmol scale) on the Applied Biosystems model 431 A peptide synthesizer, starting with Rink amide MBHA resin. Assemblies of peptides and glycopeptides **I, V, VIII, X**, and **XI** were performed essentially by the procedure already described for the preparation of **III** and **VI**.[15] The Fmoc strategy and a single coupling protocol with DCC/HOBT in NMP was used throughout all syntheses. Coupling yields (>99.6%) were determined by ninhydrin analysis.[16] The possible intrachain aminolysis at the dipeptide stage[17] was prevented by using, in the acylation step, a dipeptide containing the third and second residues [Fmoc-Tyr-(tBu)-Pro-OH for **III** and Fmoc-Tyr(tBu)-Hyp-OH for **V**]. Final yields were in the range 58−62%. The amino acid composition of the peptides was verified by amino acid analysis.

Receptor Affinity and in Vitro Biological Activity. Opioid receptor affinities of the synthesized peptides are expressed by the inhibition constant (k_i) of the binding of selective radioligands to mice brain membrane preparations: the μ- and δ-opioid receptor binding sites were selectively labeled using [^{3}H][D-Ala^2,MePhe^4,-Glyol^5]enkephalin (DAGO) and [^{3}H]naltrindole (NLT), respectively. The in vitro biological activity was tested in two isolated smooth muscle preparations: guinea pig ileum (GPI) and mouse vas deferens (MVD) that are rich in μ- and δ-opioid receptors, respectively (Table 3).

Substitution of Ser[7] with Ala in the dermorphin sequence did not affect the μ-receptor affinity and the biological potency on GPI preparations. μ-Affinity and potency were slightly reduced by glycosylation of the C-terminal residue (peptides **IV** and **VII**) but significantly reduced (5−9 times) when the sugar was fully acetylated (peptides **III** and **VI**). Biological activity on

† Abbreviations: Ac, acetyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; Fmoc, 9-fluorenylmethyloxycarbonyl; Gal, D-galactopyranose; Glc, D-glucopyranose; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; NMP, *N*-methylpyrrolidone; tBu, *tert*-butyl; TFA, trifluoroacetic acid.

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Table 1. Glycosylated Dermorphin and [D-Ala^2,Ser^7]Deltorphin I Analogues Prepared by the Solid-Phase Procedure

peptide	no.
H-Tyr-D-Ala-Phe-Asp-Val-Val-[βGlc(Ac)$_4$]Ser-NH$_2$	**I**
H-Tyr-D-Ala-Phe-Asp-Val-Val-(βGlc)Ser-NH$_2$	**II**
H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-[βGlc(Ac)$_4$]Ser-NH$_2$	**III**
H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-(βGlc)Ser-NH$_2$	**IV**
H-Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Lys-NH$_2$	**V**
H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-[αGal(Ac)$_4$]Ala-NH$_2$	**VI**
H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-(αGal)Ala-NH$_2$	**VII**
H-Tyr-D-Ala-Phe-Asp-Val-Val-[αGal(Ac)$_4$]Ala-NH$_2$	**VIII**
H-Tyr-D-Ala-Phe-Asp-Val-Val-(αGal)Ala-NH$_2$	**IX**
H-Tyr-D-Ala-Phe-Asp-Val-Val-Ser-NH$_2$	**X**
H-Tyr-D-Ala-Phe-Asp-Val-Val-Ala-NH$_2$	**XI**

MVD preparations and δ-opioid receptor affinity are not affected by β-O-glucosylation, but α-C-galactosylation reduced 2−3 times the receptor affinity and the biological potency of [Ala^7]dermorphin**.** [Hyp^6,Lys^7]dermorphin (peptide **V**) displayed both μ-receptor affinity and biological potency comparable to dermorphin.

Substitution of Gly^7 with Ser or Ala in the deltorphin I molecule reduced both δ-receptor affinity and biological potency on MVD preparations. β-O-Glucosylation of [Ser^7]deltorphin I (peptide **II**) did not produce further alterations, but α-C-galactosylation (peptide **IX**) reduced the δ-receptor affinity by a factor of 4 and the biological potency of [Ala^7]deltorphin I by a factor of 5−6. These results further support the observations on the importance of the C-terminal sequence to address the molecule toward the δ-receptor.[18,19] As previously suggested by NMR data,[20−22] maximal affinity and selectivity for δ-opioid receptors are achieved by S-shaped backbone structures in which the distance between Tyr^1 and Phe^3 rings is about 8 Å and the turn conformation of the C-terminal tripeptide is roughly coplanar with the conformation of the N-terminal sequence. On the contrary, μ-receptor recognition is attained by topologies in which the aromatic rings Tyr^1 and Phe^3 are at a distance greater than 12−13 Å and in which the bulk of the hydrophobic C-terminal domain extends away from the average plane of the rest of the molecule. The substitution of the last amino acid in the deltorphin sequence produced an appreciable decrease (20−200 times) in the δ-receptor affinity but had practically no effect on the μ-receptor affinity. Apparently the effect of substitution of Gly^7 in the deltorphin sequence on the affinity for δ-opioid receptors was related to the variation in the hydrophobicity and bulkiness of the C-terminal domain. Comparison of the HPLC retention times indicated a very similar hydrophobicity for peptides **II**, **IX**, **X**, and **XI**.

Peptide Degradation. The decay of biological activity of glycosylated dermorphin and deltorphin analogues in mouse brain and liver homogenates is shown in Table 4. The degradation profiles observed suggested that glycosylation and, even more, acetylglycosylation of the seventh amino acid residue in the dermorphin and deltorphin molecule delayed the decay of biological activity, affording protection against enzymatic breakdown. This effect was more evident in liver than in brain homogenates.

Antinociception. Antinociception was evaluated after subcutaneous administration of the synthesized glycopeptides, in mice, with the hot-plate test. The test measures the amount of time required for mice to react to standardized noxious stimuli. Since the hot-plate response has been interpreted to require the activation of supraspinal mechanisms, elongation of the reaction time supports a site of action within the brain for the active compounds. Dermorphin and its glycosylated analogues elicited a dose-dependent antinociception: their analgesic potency in mice followed that previously obtained in rats.[14] The rank order of analgesic potency was [(αGal)Ala^7]dermorphin > [βGlc]dermorphin > dermorphin > [βGlc(Ac)$_4$]dermorphin > [αGal(Ac)$_4$-Ala^7]-dermorphin (see Table 3). It is noteworthy that the glycodermorphins were at least 2 times more potent than dermorphin despite the lower μ-opioid receptor affinity, and the acetylated glycodermorphins were only 2−3 times less potent than dermorphin although they displayed a μ-opioid receptor affinity 8−10 times lower than dermorphin. Glycodermorphin's antinociception was antagonized by pretreatment with a low dose (0.1 mg/kg, sc) of the μ-selective opioid receptor antagonist naloxone. [Hyp^6,Lys^7]dermorphin (peptide **V**) was about 3 times more potent than dermorphin, by peripheral route, although the polar hydroxyproline and lysine residues increased the hydrophilicity of the compound. Substitution of Gly^7 in the deltorphin I molecule with Ser or Ala reduced 37 and 50 times the δ-receptor affinity and 21 and 35 times the biological potency on MVD (see Table 3): sc administration in mice of [Ser^7]-deltorphin and [Ala^7]deltorphin (up to 100 μmol/kg) failed to induce any significant antinociception. However, [(βGlc)Ser^7]deltorphin (AD_{50} = 44 μmol/kg) and [βGlc(Ac)$_4$)-Ser^7]deltorphin (AD_{50} = 70 μmol/kg), whose δ-affinity was comparable to that of the unglucosylated peptide, produced dose- and time-related antinociception. [(αGal)Ala^7]deltorphin (AD_{50} = 19 μmol/kg) and [αGal(Ac)$_4$)-Ala^7]deltorphin (AD_{50} = 61 μmol/kg), with δ-affinity 4−6 times lower than that of the ungalactosylated peptide, were even more potent analgesics, showing that the bioavailability to the CNS dramatically depends on the α-C-linked galactosyl residue (Table 3). It is worthy of note that [(αGal)Ala^7]deltorphin, as a peripheral antinociceptive agent, was 4 times more potent than deltorphin I (AD_{50} = 87 μmol/kg): considering that its affinity and potency were about 180 times lower than those of deltorphin I, it follows that the CNS bioavailability of [(αGal)Ala^7]deltorphin must be about 700 times higher than that of deltorphin I. Antinociception induced by 20 μmol/kg, sc, of [(αGal)Ala^7]deltorphin (%MPE = 54 ± 7) was antagonized (%MPE = 9 ± 2) by pretreatment with very a low dose (0.3 mg/kg, sc) of the δ-selective antagonist naltrindole,[22] confirming that glycodeltorphin analgesia depends on selective activation of the δ-opioid system.

In conclusion, glycosylation of the seventh amino acid residue of dermorphin and deltorphin I yielded molecules endowed with higher analgesic potency than their parent compounds when sc injected in mice. It might be explained by the protection toward enzymatic degradation afforded by glycosylation (Table 4) and the consequent increase in the peptide blood and CNS concentration. Glycosides, which behaved as the most potent analgesics, are highly polar compounds. Acetylation of the sugar hydroxyl functions increased lipophilicity as well as the resistance to enzymatic degradation but did not increase the analgesic potency after

Table 2. Optical Rotation and Amino Acid Ratios of the Indicated Peptides[a]

		amino acid ratio									
no.	$[\alpha]^{21}_D$ (*c* 1.0, H_2O)	Asp	Ser	Pro	G1y	Ala	Val	Tyr	Phe	Lys	Hyp
I	−18.5°	1.02	1.00			1.00	1.98	0.98	1.02		
II	−0.4°	1.02	nd			1.03	1.99	0.95	1.00		
III	−5.4°		0.99	1.03	1.01	0.99		1.94	1.01		
IV	+0.8°		1.02	1.02	1.02	1.00		1.96	1.00		
V	+0.7°				1.00	1.02		1.99	1.02	0.98	0.99
VI	+39.9°			1.01	1.01	1.01		1.94	1.03		
VII	+36.7°			1.00	1.07	1.02		1.93	1.02		
VIII	+16.2°	nd				1.00	1.99	1.00	1.00		
IX	+11.2°	nd				0.99	2.00	0.99	1.01		
X	−1.1° *	1.04	0.9			1.05	1.95	1.02	1.04		
XI	−3.8° *	1.00				2.05	1.97	0.99	0.99		

[a] The values denote the number of residues per molecule; nd, not determined; *c = 0.5, acetic acid.

Table 3. μ- and δ-Opioid Receptor Affinities and Biological Activities of Glycopeptides[a]

	$K_{i,\ nM}$		IC_{50}, nM		AD_{50}, μmol/kg
peptide	μ [^{3}H]DAGO	δ [^{3}H]NLT	GPI	MVD	analgesia hot-plate test
dermorphin	1.1 ± 0.2	1004 ± 98	1.5 ± 0.5	19 ± 3	2.4 (1.7−3.4)
[(βGlc)Ser^7]dermorphin (**IV**)	2.4 ± 0.3	1250 ± 150	3.5 ± 0.4	40 ± 5	1.4 (1.2−1.6)*
[βGlc(Ac)$_4$-Ser^7]dermorphin (**III**)	7.9 ± 0.9	1320 ± 147	8.2 ± 0.7	18 ± 2.9	4.6 (3.0−6.9)
[Ala^7]dermorphin	1.2 ± 0.2	1111 ± 139	1.6 ± 0.2	24 ± 2	nt
[(αGal)Ala^7]dermorphin (**VII**)	2.5 ± 0.4	2850 ± 230	4.0 ± 0.5	55 ± 4.5	1.1 (0.9−1.2)*
[αGal(Ac)$_4$-Ala^7]dermorphin (**VI**)	11.2 ± 2.1	> 5000	6.0 ± 0.5	65 ± 7.1	6.6 (6.5−6.7)
[Hyp^6,Lys7]dermorphin (**V**)	0.5 ± 0.2	1411 ± 200	2.1 ± 0.2	35 ± 4.1	0.9 (0.7−1.1)*
deltorphin I	1980 ± 220	0.8 ± 0.1	1239 ± 203	0.2 ± 0.03	87 (35−211)
[Ser^7]deltorphin (**X**)	1100 ± 125	30 ± 3.9	1400 ± 107	4.3 ± 0.5	*b*
[(βGlc(Ser^7]deltorphin (**II**)	879 ± 100	23 ± 1.9	1600 ± 133	3.1 ± 0.4	44 (32−61)
[βGlc(Ac)$_4$-Ser^7]deltorphin (**I**)	1915 ± 210	28 ± 2.1	1900 ± 203	2.7 ± 0.4	70 (54−91)
[Ala^7]deltorphin (**XI**)	1898 ± 194	40 ± 3.5	>2000	6.9 ± 0.5	*b*
[(αGal)Ala^7]deltorphin (**IX**)	1586 ±163	176 ± 19	>2000	36 ± 4.1	19 (10−31)**
[αGal(Ac)$_4$-Ala^7]dermorphin (**VIII**)	2827 ± 201	160 ± 15	>2000	43 ± 4.7	61 (35−106)

[a] K_i, dissociation constant; the values are the mean of three experiments ± SEM. μ-Opioid receptors were labeled with [^{3}H]DAGO, 0.5 nM; δ-opioid receptors were labeled with [^{3}H]NLT, 0.1 nM. IC_{50}, agonist concentration that produced 50% inhibition of the electrically evoked twitch; the values are the mean of six experiments ± SEM. AD_{50}(CL), median antinociceptive dose and 95% confidence limits. *P < 0.05 vs dermorphin; **P < 0.05 vs deltorphin; nt, not tested. [b] The highest dose tested, 100 μmol/kg, was unable to produce a significant analgesia.

Table 4. Half-Time of Enzymatic Breakdown of Dermorphin and Deltorphin Analogues in Mouse Brain and Liver Homogenates

	$t_{1/2}$ (min)	
peptides	brain	liver
dermorphin	20 ± 5	10 ± 4
[(βGlc)Ser^7]dermorphin	38 ± 6	30 ± 5
[βGlc(Ac)$_4$-Ser^7]dermorphin	90 ± 10	60 ± 8
[Hyp,^{6}Lys^7]dermorphin	30 ± 5	20 ± 4
deltorphin I	240 ± 15	110 ± 20
[(βGlc)Ser^7]deltorphin	>240 (70%)[a]	180 ± 25
[βGlc(Ac)$_4$-Ser^7]deltorphin	>240 (87%)[a]	>240 (70%)[a]

[a] Numbers in parentheses are the residual biological activity after a 240-min incubation.

peripheral administration. In situations where increased hydrophilicity does not hinder but even improves diffusion across the blood−brain barrier, the presence of specialized transport processes must be considered. The GLUT-1 transporter, suggested by Polt et al.[2] for the glucosylated enkephalins, shows a selective preference for the β-O-links and a 3 times higher affinity for glucose than for galactose. Thus the expectation was that, by peripheral administration, the galactosylated derivatives were far less potent analgesics than the glucosylated ones. On the contrary, [(αGal)Ala^7]-dermorphin was slightly more analgesic than [βGlc]-dermorphin, even if it displayed comparable μ-receptor affinity, and [(αGal)Ala^7]deltorphin was at least 2 times more analgesic than [(βGlc)Ser^7]deltorphin, even if it had 7 times lower δ-receptor affinity. Different carrier-mediated transport systems have been demonstrated for some opioid peptides[24,25] and, particularly, for deltorphin.[26] Moreover, we cannot exclude that the high polarity afforded to the peptides by the glycosyl residue or by hydrophilic amino acids (as Hyp-Lys) activates some kind of endocytosis.[27,28] Whatever the transport system, glucosylation and, even more, galactosylation of dermorphin and deltorphin I yielded analogues possessing remarkable antinociceptive activity, by peripheral injection. In particular, the development of systemically available δ-selective opioid agonists may offer therapeutic advantage over currently available opioid analgesics. The systemic active opioid δ-agonists available at the present time are nonpeptidic compounds that, despite a significant selectivity for δ-receptors shown in the bioassays, suggested in vivo a mixed profile of actions at μ- and δ-receptors and produced more or less toxicity[29] probably depending on unknown metabolites.

Experimental Procedures

Chemical Syntheses. Fmoc-Tyr(tBu)-OH, Fmoc-D-Ala-OH, Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Gly-OH, and Rink amide MBHA resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamido-norleucyl-4-methylbenzhydrylamine polystyrene, substitution 0.52 mmol/g] were Novabiochem products. Fmoc-[βGlc(Ac)$_4$]Ser-OH[30] and Fmoc-[αGal(Ac)$_4$]Ala-OH[31] were synthesized according to the literature, Fmoc-Tyr(tBu)-Pro-OH and Fmoc-Tyr-

(tBu)-Hyp-OH were prepared as described for the preparation of Fmoc-Pro-Hyp(tBu)-OH.[32] All other chemicals for the solid-phase synthesis were supplied by Applied Biosystems. The final peptide-resins were deprotected at the amino terminus with 20% piperidine in NMP for 20 min, prior to cleavage with aqueous 95% TFA for 90 min. The resin was removed by filtration and excess *tert*-butyl methyl ether was added to the filtrate. The precipitated peptide was collected, reprecipitated three times from aqueous 95% TFA with *tert*-butyl methyl ether, and dried. Deacetylation was achieved by adding excess hydrazine hydrate to a methanolic solution of the crude peptide (**I, III, VI**, or **IX**) (typically 30−40 μmol). After the mixture stirred for 2 h, *tert*-butyl methyl ether was added, and the resulting precipitate was collected and dried. Average deacetylation yield was 90%. Optical rotations were determined with a Perkin-Elmer model 241 polarimeter. Amino acid analyses were done with a Carlo Erba model 3A 28 amino acid analyzer equipped with a Perkin-Elmer Sigma 10 chromatography data station following hydrolysis for 22 h (46 or 70 h for determining the Val content) at 110 °C, in sealed, evacuated vials in constant boiling hydrochloric acid containing 0.2% phenol. Reverse-phase analytical HPLC separations were performed on a Perkin-Elmer series 410 liquid chromatograph equipped with a LC-90 UV detector and LCI-100 integrator (column Aquapore Octyl RP-300 222 × 4.6 mm; Brownlee Laboratories; load 10 μg, flow rate 1.5 mL/min). Eluants: A, aqueous 0.1% TFA; B, 0.1% TFA in 90% aqueous acetonitrile. Elution: isocratic 10% B for 2 min, linear gradient 10−90% B in 30 min, isocratic 90% B for 2 min. Solvents were freshly distilled and dried, and evaporations were carried out under reduced pressure, at 20−30 °C, using a rotary evaporator. Yields are based on the weight of vacuum-dried product. Optical rotations and amino acid analysis of the synthesized peptides and glycopeptides are shown in Table 2.

Binding Assays. Binding of the peptides to δ- and μ-opioid receptors was assayed on crude membrane preparations from adult male mouse brain (Albino Swiss, 35−40 g) as previously described.[18] The brains (including cerebella) were homogenized in 50 volumes of Tris-HCl buffer (50 mM, pH 7.4, 4 °C) using a Kinematica PT 3000 polytron (20 s, speed 16 000 rpm). The homogenate was centrifuged at 41000*g* for 20 min at 4 °C; pellets were resuspended in 50 vol of buffer and incubated at 25 °C per 30 min to remove endogenous opioids. After centrifugation, pellets were resuspended in buffer, containing 5% glycerol, to give a final w/v of 20 mg/mL fresh tissue. The affinity of the compounds for μ- and δ-receptors was determined by causing unlabeled compounds to compete against the μ-selective receptor ligand [^{3}H][D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin ([^{3}H]DAGO; 0.5 nM, 55.3 Ci/mmol; Amersham, U.K.) or the δ-receptor selective ligand [^{3}H]naltrindole (0.1 nM, 33 Ci/mmol; NEN, Boston, MA). Assays were carried out on 1 mg of brain membrane protein, in a final volume of 2 mL of Tris-HCl buffer (50 mM, pH 7.4), at 35 °C for 90 min. All experiments were performed in triplicate with total binding, nonspecific binding (10 μM naloxone), and 12 inhibitor concentrations determined. Reagents and membranes were distributed with a robotic sample processor (Tecan RSP 5000-series). Using a Brandel M-24 cell harvester, assays were terminated by filtration through Whatman GF/B filter strips previously soaked in 0.5% poly(ethylenimine) for 1 h. Filters were washed three times with 4 mL of ice-cold buffer and radioactivity was counted in a liquid scintillation spectrometer (Betamatic, Kontron). 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.[33] Data obtained from four independent measurements are presented as the arithmetic mean ± SEM.

Pharmacological Assays in Isolated Tissues. 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.[15] Agonists were evaluated for their inhibition of the electrically evoked twitch. The results are expressed as the IC_{50} values obtained from concentration−response curves.[34] IC_{50} values represent the mean of not less than six tissue samples ± SEM.

Test of Peptide Degradation. Peptides were incubated with mouse brain and liver homogenates in 50 mM Tris-HCl, pH 7.4, at 37 °C for 0−240 min (200 nmol of peptide/100 mg of tissue/mL). At the end of each incubation (0, 5, 10, 20, 40, 60, 120, 240 min), an equal volume of methanol was added to each sample to precipitate proteins and stop enzymatic activity. Samples were than centrifuged at 13000*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 or MVD preparations and expressed as percent of the recovery at time 0 (control). The results are mean ± SEM of three experiments.

Antinociception Studies. Male CD-1 mice (25−30 g) 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 to 18 h. The IASP guidelines on ethical standards for investigations of experimental pain in animals were followed. Compounds were dissolved in sterile Ringer's solution and administered to the mice in a volume of 0.05 mL/10 g of body weight. Every dose of each peptide was evaluated in groups of 5−8 animals. Antinociception was assessed by the hot-plate test: mice were placed on a 55 °C heated surface, and the mean time to licking of the back paws or to an escape jump was recorded. Percent antinociception was calculated as [(test latency − control latency)/(15 − control latency)] × 100 (%MPE). To avoid tissue damage, a maximal score was assigned to animals not responding within 15 s. Mice not responding within 5 s in the predrug control were excluded from further study. The test was performed before drug treatment (control) and every 15 min after drug injections, during the first hour, and every 30 min thereafter, until analgesia disappeared. The dose−response curves were analyzed via linear regression with the computer program of Tallarida and Murray:[34] the median antinociceptive dose and 95% confidence limits [AD_{50}(CL)] were calculated according to the method of Litchfield and Wilcox.

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