Effect of Modification of the Basic Residues of Dynorphin A-(1-13) Amide on κ Opioid Receptor Selectivity and Opioid Activity†,‖

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A series of dynorphin A-(1-13) amide (Dyn A-(1-13)NH_2) analogues containing lysine or N^{ϵ}-acetyllysine (Lys(Ac)) was prepared by solid-phase peptide synthesis and evaluated for opioid receptor affinity in radioligand binding assays and for opioid activity in the guinea pig ileum (GPI). Substitutions were made at positions 6, 7, 9, 11, and 13, the basic amino acids in the C-terminus of the peptide, in order to assess the individual contributions of these residues to the κ opioid receptor affinity and selectivity of Dyn A-(1-13)NH_2. While substitutions of Lys(Ac) for Arg in position 6 did not affect κ receptor affinity, it enhanced affinity for μ and δ receptors and therefore caused a loss of κ receptor selectivity. When Lys(Ac) was substituted for Arg^9, κ opioid receptor affinity was enhanced and κ receptor selectivity was retained. Replacement of Arg^7, Lys^{11}, or Lys^{13} by Lys(Ac) resulted in both decreased affinity and selectivity for κ receptors. These results demonstrate the importance of Arg^6 to the receptor selectivity profile of Dyn A-(1-13)NH_2 and indicate that, of the five basic residues in the C-terminus, only Arg^9 can be replaced by a nonbasic residue without substantial loss of κ opioid receptor selectivity.

Introduction

Dynorphin A (Dyn A), originally isolated from porcine pituitary,[1] is postulated to be an endogenous κ opioid receptor ligand.[2] Dyn A shares a common N-terminal tetrapeptide sequence (the "message" sequence) with other mammalian opioid peptides, while containing a unique C-terminal "address" sequence which imparts selectivity for κ opioid receptors.[3] The shortened Dyn A-(1-13), with the sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, accounts for essentially all of the biological activity of Dyn A in the guinea pig ileum (GPI) assay.[4]

Shortly after the determination of the sequence of Dyn A-(1-13), studies were undertaken to determine which amino acids in the C-terminal "address" region of the peptide are important for opioid potency and κ receptor selectivity. The first of these involved successive truncation of Dyn A-(1-13) from the C-terminus and the evaluation of the fragments in the GPI.[3] This study found that the basic residues Arg^7, Lys^{11}, and Lys^{13} were important residues for potency. Based upon the sensitivity of the fragments to antagonism by naloxone, it was concluded that Arg^7 and Lys^{11} made the greatest contributions to κ receptor selectivity. In another study, Dyn A and related fragments Dyn A-(1-13), -(1-11), -(1-9), -(1-7), and -(1-5) were tested in ileum preparations made tolerant to morphine or ethylketocyclazocine.[5] While the extension of Leu-enkephalin by the two basic residues in positions 6 and 7 conferred some dynorphin-like properties to the peptide, the longer fragments Dyn A-(1-11) and Dyn A-(1-13) exhibited the greatest preference for κ receptors. Because fragments were used in both of these studies, however, the contributions of individual C-terminal residues to κ opioid receptor selectivity and potency could not be separated from the effects of previously truncated residues. In particular, the contribution of Arg^6 to κ receptor selectivity could not be determined, since truncation of the important Arg^7 residue results in a peptide, Dyn A-(1-6), which no longer exhibits selectivity for κ receptors.

The contribution of individual residues to potency and receptor selectivity can be assessed by amino acid substitution in the peptide. In one study, alanine was systematically substituted for each amino acid in positions 1-11 in Dyn A-(1-13).[6] The analogues were evaluated for opioid activity in smooth muscle assays and for opioid receptor affinity in a [^{3}H]etorphine binding assay in rat brain. From this study the most important sites in the address portion for potency appeared to be Arg^6 and Arg^7, with Arg^9 and Lys^{11} making smaller contributions. No information was reported, however, on what effects these substitutions had on selectivity for κ receptors.

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‖ Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977-983. Amino acids are in the L-configuration. Additional abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,MePhe^4,Glyol]enkephalin; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Lys(Ac), N^{ϵ}-acetyllysine; MBHA, 4-methylbenzhydrylamine; Pmc, 2,2,5,7,8-pentamethylchromanyl-6-sulfonyl; TFA, trifluoroacetic acid.

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We are interested in preparing a variety of Dyn A analogues, including conformationally constrained derivatives, with enhanced selectivity for κ opioid receptors. While the contributions of Arg[7] and Lys[11] to Dyn A's potency and selectivity are generally accepted, the relative importance of the basic residues in positions 6 and 9 are less clear. Thus these latter two positions could be potential sites for incorporation into a conformational constraint or other structural modification. Incorporation into a conformational constraint such as a lactam or cyclic disulfide would entail the loss of the residue's basicity. Therefore we investigated whether either of these positions could be replaced by a nonbasic residue by preparing linear analogues containing N^{ϵ}-acetyllysine (Lys(Ac)) in each of these positions. We also substituted Lys(Ac) for the basic residues in positions 7, 11, and 13 in Dyn A-(1–13)NH_2. This allowed us to evaluate the relative importance for κ opioid receptor selectivity and potency of residues 6 and 9 as compared to residues 7, 11, and 13. We used the peptide amide because Dyn A-(1–13)NH_2 retains the κ receptor selectivity of the corresponding acid and is more stable to enzymatic degradation.[7] Since arginine is normally present in positions 6, 7, and 9 in Dyn A-(1–13), the analogues containing lysine in these positions were also prepared to allow for a more direct comparison to the acetylated analogues.

Results

Peptide Synthesis and Purification. All peptides were synthesized on a PAL resin (a 4-methylbenzhydrylamine (MBHA) resin with a 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy]valeric acid linker[8]) using (9-fluorenylmethoxy)carbonyl (Fmoc) protected amino acids. Side-chain protecting groups used in this synthetic strategy were *tert*-butyloxycarbonyl (Boc) for Lys, 2,2,5,7,8-pentamethylchromanyl-6-sulfonyl (Pmc)[9] for Arg, and *tert*-butyl (tBu) for Tyr. The Fmoc-protected amino acids were coupled to the growing peptide chain using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in DCM/DMF or DCM/DMA (1/1). The Fmoc protecting group was removed with 30% piperidine in DMF/toluene or DMA/toluene (1/1). The peptides were deprotected and cleaved from the PAL resin using concentrated trifluoroacetic acid (TFA) in the presence of scavengers (generally Reagent K[10]) and purified by preparative reverse-phase HPLC. The purified peptides were characterized by analytical HPLC, fast atom bombardment mass spectrometry (FAB-MS), and amino acid analysis (Tables I and II).

Opioid Receptor Binding Affinities and Opioid Activity. The peptides were evaluated for opioid receptor affinity at κ, μ, and δ receptors by measuring the inhibition of binding of [^{3}H]bremazocine, [^{3}H]DAMGO ([D-Ala^2,-MePhe^4,Glyol]enkephalin),[11] and [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin),[12,13] respectively (Table III), as previously described.[14] κ opioid receptor affinity was determined in the guinea pig cerebellum, since over 80% of the opioid receptors in this tissue are κ receptors.[15] μ and δ opioid receptor affinities were assessed in rat forebrain membranes. The opioid activity of the peptides was determined in the electrically-stimulated GPI[14] (Table IV).

Table I. Yields, HPLC Analysis, and Fast-Atom Bombardment Mass Spectrometry (FAB-MS) Analysis of [Lys]- and [Lys(Ac)]Dyn A-(1–13)NH_2 Analogues

Dyn A-(1–13)NH_2 analogue	% yield	HPLC gradient:[a] R_v, mL	HPLC isocratic: R_v, mL (% B)[b]	FAB-MS: M + 1
Lys[6]	37	31.4	19.8 (16)	1575
Lys(Ac)[6]	45	30.0	16.8 (16)	1617
Lys[7]	8[c]	29.4	11.9 (17)	1575
Lys(Ac)[7]	49	30.2	11.2 (19)	1617
Lys[9]	81	27.9	16.2 (18)	1575
Lys(Ac)[9]	56	30.5	22.2 (18)	1617
Lys(Ac)[11]	26	30.6	7.5 (15)	1645
Lys(Ac)[13]	40	30.1	11.9 (19)	1645

[a] 0–75% B over 50 min at 1.5 mL/min. [b] Solvent composition indicated in parentheses. [c] Yield low due to procedural losses during purification; see Experimental Section.

Replacement of the arginines at positions 6, 7, and 9 in Dyn A-(1–13)NH_2 by lysine generally caused the greatest changes in receptor affinity at μ receptors, where receptor affinity was enhanced 5–22-fold compared to Dyn A-(1–13)NH_2 (Table III). The affinity for κ receptors was unchanged when the arginines at positions 6 and 7 were replaced by lysine, while substitution of lysine for Arg[9] increased κ receptor affinity 3-fold when compared to the parent peptide. The net result was a loss of discrimination between κ and μ receptor binding for all three of the analogues. The substitution of lysine in positions 7 and 9 had little effect on the analogues' affinity for δ receptors, while [Lys[6]]Dyn A-(1–13)NH_2 exhibited decreased affinity for δ opioid receptors. [Lys[7]]Dyn A-(1–13)NH_2 thus showed discrimination between κ and δ opioid receptors similar to that of the parent peptide, while [Lys[6]]- and [Lys[9]]Dyn A-(1–13)NH_2 showed improved discrimination between κ and δ receptors compared to Dyn A-(1–13)NH_2.

The positive charge at each of positions 6, 7, 9, 11, and 13 was removed by acetylating the side-chain amine of lysine. Unlike replacement of Arg[6] by Ala, which decreases opioid receptor affinity in rat brain,[6] acetylation of lysine in position 6 had little effect on κ receptor affinity. It enhanced μ binding affinity, however, causing a complete loss of κ receptor selectivity. When the 7 position was acetylated, κ binding showed a 6-fold decrease while μ binding decreased only 3-fold, so discrimination between

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Table II. Amino Acid Analysis of [Lys]- and [Lys(Ac)]Dyn A-(1–13)NH_2 Analogues

analogue	Y(1)	G(2)	F(1)	L(2)	R(2/3)	I(1)	P(1)	K(2/3)
Lys^6	0.95	2.00	0.98	2.03	2.10	0.93	1.03	3.01
$Lys(Ac)^6$	0.86	2.03	0.96	1.96	2.12	0.86	1.21	3.02
Lys^7	0.88	1.94	0.96	2.10	2.01	1.08	1.18	2.85
$Lys(Ac)^7$	0.99	1.97	0.98	1.97	2.07	0.95	1.12	2.97
Lys^9	0.89	2.03	1.00	2.14	2.04	1.07	1.06	2.78
$Lys(Ac)^9$	0.87	2.06	0.98	2.14	2.04	1.04	1.09	2.79
$Lys(Ac)^{11}$	0.89	2.06	0.99	2.18	2.94	1.00	1.04	1.91
$Lys(Ac)^{13}$	1.02	1.96	1.01	1.98	3.07	0.93	1.02	1.99

Table III. Opioid Receptor Binding Affinities and Selectivities of [Lys]- and [Lys(Ac)]Dyn A-(1–13)NH_2 Analogues

peptide	K_i (nM) [^{3}H]bremazocine	[^{3}H]DAMGO	[^{3}H]DPDPE	receptor selectivity ($\kappa/\mu/\delta$)[a]
Dyn A-(1–13)NH_2	0.023 ± 0.009	0.40 ± 0.06	1.9 ± 0.2	1/18/83
Lys^6	0.020 ± 0.005	0.075 ± 0.018	5.9 ± 1.7	1/3.9/300
$Lys(Ac)^6$	0.024 ± 0.003	0.018 ± 0.003	0.42 ± 0.11	1.3/1/23
Lys^7	0.021 ± 0.001	0.055 ± 0.006	1.6 ± 1.4	1/2.6/78
$Lys(Ac)^7$	0.14 ± 0.06	0.18 ± 0.02	1.1 ± 0.2	1/1.3/7.7
Lys^9	0.0067 ± 0.0010	0.046 ± 0.005	1.9 ± 0.4	1/6.9/280
$Lys(Ac)^9$	0.0042 ± 0.0013	0.043 ± 0.008	1.3 ± 0.2	1/10.4/310
$Lys(Ac)^{11}$	0.053 ± 0.017	0.030 ± 0.001	0.45 ± 0.27	1.8/1/15
$Lys(Ac)^{13}$	0.15 ± 0.06	0.061 ± 0.004	4.6 ± 0.7	2.4/1/75

[a] Ratio of K_is where the lowest K_i is used as the denominator.

Table IV. Opioid Activity of [Lys]- and [Lys(Ac)]Dyn A-(1–13)NH_2 Analogues in the Guinea Pig Ileum

Dyn A-(1–13)NH_2 analogue	IC_{50}, nM (95% confidence interval)	rel potency (%)
Dyn A-(1–13)NH_2	0.24 (0.21–0.29)	100
Lys^6	0.25 (0.13–0.50)	94
$Lys(Ac)^6$	0.64 (0.31–1.34)	37
Lys^7	0.19 (0.13–0.29)	122
$Lys(Ac)^7$	0.34 (0.26–0.45)	69
Lys^9	0.27 (0.19–0.37)	89
$Lys(Ac)^9$	0.26 (0.18–0.38)	91
$Lys(Ac)^{11}$	0.16 (0.12–0.21)	148
$Lys(Ac)^{13}$	0.19 (0.14–0.24)	126

the two receptor types was again nonexistent. Binding affinity for κ receptors decreased 2-fold and 6-fold for [$Lys(Ac)^{11}$]- and [$Lys(Ac)^{13}$]Dyn A-(1–13)NH_2, respectively, while the affinity of these peptides for μ receptors increased 6–13-fold, so that they exhibited a slight preference for μ over κ receptors. The one exception to this loss of κ selectivity was [$Lys(Ac)^9$]Dyn A-(1–13)NH_2, which showed enhanced κ receptor affinity and selectivity compared to its parent, [Lys^9]Dyn A-(1–13)NH_2, and was the most κ selective of the Dyn A-(1–13)NH_2 analogues prepared. The marked enhancement in affinity for κ and μ receptors by [$Lys(Ac)^9$]Dyn A-(1–13)NH_2 over Dyn A-(1–13)NH_2 is in contrast to the modest decrease in affinity observed for [Ala^9]Dyn A-(1–13) in [^{3}H]etorphine binding assay in rat brain.[6]

Acetylation in the 6, 7, and 11 positions enhanced affinity for δ receptors, and thus decreased the discrimination between κ and δ receptors for these peptides. Interestingly, acetylation of Lys^{13} decreased affinity for δ opioid receptors, but because of this analogue's decreased affinity for κ receptors its discrimination between κ and δ receptors was very similar to that of Dyn A-(1–13)NH_2. While acetylation at the 9 position slightly increased δ opioid receptor affinity, the marked enhancement in the κ receptor affinity of [$Lys(Ac)^9$]Dyn A-(1–13)NH_2 increased its discrimination for κ over δ receptors, similar to its parent peptide [Lys^9]Dyn A-(1–13)NH_2.

All of the analogues exhibited subnanomolar potency in the guinea pig ileum (Table IV). Only [$Lys(Ac)^6$]Dyn A-(1–13)NH_2 showed a significant decrease in opioid activity when compared to either its parent peptide [Lys^6]-Dyn A-(1–13)NH_2 or Dyn A(1–13)NH_2. [Lys^9]- and [$Lys(Ac)^9$]Dyn A-(1–13)NH_2, which were very potent analogues in the κ and μ binding assays, exhibited potency similar to Dyn A-(1–13)NH_2 in the GPI. Overall, the replacement of the basic residues by Lys(Ac) was better tolerated than replacement by Ala, which caused at least a 7-fold decrease in activity in the GPI.[6] The antagonism of selected analogues by norbinaltorphimine, a κ-selective antagonist,[16,17] was examined in order to evaluate whether these peptides were acting on κ receptors in the GPI. Norbinaltorphimine at a dose of 10 nM consistently shifted the dose–response curves of [Lys^6]-, [$Lys(Ac)^6$]-, [Lys^9]-, [$Lys(Ac)^9$]Dyn A-(1–13)NH_2 and Dyn A-(1–13)NH_2 20–30-fold, indicating that the agonist activity of these compounds in the GPI is mediated at least in part by κ receptors.

Conclusions and Discussion

The above results have clear implications for the design of future Dyn A analogues, particularly the design of conformationally constrained peptides. Our results for [$Lys(Ac)^6$]Dyn A-(1–13)NH_2 demonstrate that Arg^6 is a key determinant in the address sequence of Dyn A for the peptide's receptor selectivity profile. As mentioned above, the importance of this residue was not revealed by the earlier studies of truncated sequences.[5,6] While a basic residue at position 6 in Dyn A is not required for κ receptor binding, it decreases the peptide's interactions with μ and δ receptors, enhancing κ selectivity. These results suggest that cyclization through this position to give conformationally constrained analogues could have an adverse affect on selectivity for κ receptors. Of the five basic residues in the address sequence, our data indicate that only Arg^9 can easily be replaced by a nonbasic amino acid without substantial loss of κ receptor selectivity, making this a logical site for incorporation into a conformational constraint. Our results for substitution of the remaining basic

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residues in positions 7, 11, and 13 are consistent with the earlier observations that these residues are important for κ receptor affinity and selectivity.[5]

Experimental Section

Materials. The reagents used in peptide synthesis were as follows: FmocPAL Resin (MBHA resin with a 5-[4-(Fmoc-aminomethyl)-3,5-dimethoxyphenoxy]valeric acid linker), HOBt and all amino acids except FmocLys and FmocLys(Ac) (Milligen/Biosearch, Novato, CA); FmocLys (Bachem, Torrence, CA); TFA (Kali-Chemie, Greenwich, CT); anisole, ethanedithiol, and DIC (Aldrich); acetic anhydride (Baker); and HPLC-grade solvents DCM, DMF, DMA, MeOH (Burdick and Jackson or Merck Omnisolv). The reagents and supplies used in purification were as follows: HPLC-grade AcCN and MeOH (Burdick and Jackson); TFA (Pierce Sequanal grade in amber ampules); HPLC-grade water (Milli-Q system, College of Pharmacy); Syrfil disposable HPLC filters, 0.45-μm pore size. Other reagents were reagent grade.

FmocLys(Ac) was prepared initially by acetylation of FmocLys with acetic anhydride (6 equiv) in DMF for 3 h, followed by evaporation, trituration with ether, and recrystallization from EtOAc. Subsequently FmocLys(Ac) was obtained from Peninsula Laboratories, Belmont, CA. The melting points (156–158 °C) and analytical HPLC (R_v = 39.5 mL, 0–75% B over 50 min) were identical for the two samples.

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA). The peptides were analyzed using a Beckman Model 431A gradient HPLC system consisting of a Model 421A controller, two Model 110B pumps, Model 201A injector, Model 163 detector, and a Waters Model 740 data module. The peptides were purified on the above system with an ISCO UA-5 UV detector or on a Rainin HPLC gradient system with HPLX pumps and the ISCO detector. The HPLC columns used were a Vydac 214TP54 C_4 analytical column (300 Å, 5 μm, 4.6 mm × 25 cm) with a Vydac C_4 214TP guard cartridge, and a Protein Plus preparative column (300 Å, 10 μm, 21 mm × 25 cm) with a Dynamax (21 mm × 5 cm, C_4, 12 μm) guard cartridge. The peptides were eluted using 0.1% TFA in H_2O (solvent A) and 0.1% TFA in AcCN (solvent B). For the analytical and preparative columns the flow rates were 1.5 mL/min and 10 or 20 mL/min, respectively, and the eluents were monitored at 214 nm (Beckman Model 163 detector) and 280 nm (ISCO UA-5 detector), respectively. The aqueous solutions were lyophilized on a Thermovac Model FD-6 lyophilizer.

The peptides were characterized by FAB-MS and amino acid analysis. The FAB-MS was performed on a Kratos MS50RF in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR. Peptide samples were hydrolyzed for 24 h at 110 °C with 6 N HCl plus 1% phenol and amino acid content determined by the Center for Gene Research and Biotechnology, Central Services Laboratory, Oregon State University, on a Beckman System Gold Model 126AA (2 × 250 mm Spherogel column), using ninhydrin detection at 570 nm.

Peptide Synthesis Using Fmoc-Protected Amino Acids. Solid-Phase Peptide Synthesis. All peptides were synthesized using the Fmoc chemical protocol on the Biosearch 9500. The FmocPAL resin (0.5 g, 0.27 mmol/g resin substitution) was washed with 5 × 20 mL of DCM/DMF (1/1) and the synthesis carried out as follows: the deprotection of the resin was performed for 3 min and then 7 min in piperidine/toluene/DMF (30/35/35, v/v), and the resin then washed with DCM/DMF (1/1, 12×). The solution of the desired Fmoc amino acid (0.4 M in DMF, 4.0-fold excess) with 1 equiv of HOBt was mixed with an equal amount of 0.4 M DIC in DCM and reacted with the resin for 2 h; the resin was then washed with DCM/DMF (1/1, 12×). Amino acids with side chain protecting groups used were FmocArg(Pmc), FmocLys(Boc), and FmocTyr(tBu). Ninhydrin was used on a small sample of resin to determine if couplings were complete. Following a coupling reaction the Fmoc group was removed as described above and the next amino acid coupled to the resin. After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, the resin was washed successively with DCM/DMF (1/1), DCM, and MeOH, collected, and dried overnight in vacuo. DMA was used in place of DMF in the synthesis of [Lys(Ac)^7]- and [Lys(Ac)^{13}]Dyn A-(1–13)NH_2.

Cleavage of the Peptide from the Support. The dried protected peptide resins were generally reacted at room temperature with 5 mL Reagent K[10] (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol) under nitrogen for 4–5 h. The peptides were filtered from the resin, washed with TFA, and concentrated to about 2–5 mL. The solutions were diluted with 10% acetic acid (40 mL) and then extracted with Et_2O (3×), and the aqueous layers lyophilized to give the crude peptides. [Lys^6]Dyn A-(1–13)NH_2 was obtained by treating the corresponding resin with 5 mL of Reagent R[8] (90% TFA, 5% thioanisole, 3% ethanedithiol, and 2% anisole) under nitrogen for 24 h, followed by filtration, concentration, and trituration of the peptide with ether. [Lys(Ac)^6]Dyn A-(1–13)NH_2 was cleaved from the resin using TFA/anisole (9/1) overnight, followed by filtration, concentration, and trituration with ether.

Purification of the Peptides. The crude peptides were purified by preparative HPLC using a mobile phase gradient of either 100% A to 50% B over 50 min at 20 mL/min or 100% A to 70% B over 140 min at 10 mL/min. [Lys^7]Dyn A-(1–13)NH_2 required a second purification by preparative HPLC. Generally peak fractions were analyzed by isocratic analytical HPLC and pure fractions combined and lyophilized. The resulting pure peptides were characterized by FAB-MS, amino acid analysis, and analytical HPLC (Tables I and II).

Binding Assays. Guinea pig cerebellar membranes and rat forebrain membranes were prepared as previously described.[14] The inhibition of the binding of [^{3}H]bremazocine to guinea pig cerebellar membranes (κ) was measured at 4 °C in the presence of bestatin as previously reported,[14] except that 100 nM DAMGO was included in the incubation mixtures. Nonspecific binding was determined in the presence of 1 μM Dyn A-(1–13)NH_2. The inhibition of the binding of [^{3}H]DAMGO (μ) and [^{3}H]DPDPE (δ) to rat forebrain membranes was measured at 4 °C in the presence of peptidase inhibitors, as previously described;[14] nonspecific binding was measured in the presence of 10 μM levorphanol and 10 μM unlabeled DPDPE, respectively. Equilibrium inhibition constants (K_is) were calculated from the Cheng and Prusoff equation,[18] using 0.0549, 0.314, and 7.63 nM for the K_D values of tritiated bremazocine, DAMGO, and DPDPE, respectively.

Guinea Pig Ileum Assay. Guinea pig ileum assays were performed as previously described.[14] In experiments involving norbinaltorphimine, the antagonist was added to the tissue bath 10 min prior to determination of the agonist dose–response curve.

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