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Bis-penicillamine enkephalins possess highly improved specificity toward δ opioid receptors

(conformational restrictions/mouse vas deferens/guinea pig ileum/rat brain receptor binding)

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ABSTRACT **The conformationally restricted, cyclic, disulfide-containing, enkephalin analogs [2-D-penicillamine, 5-L-penicillamine]enkephalin ([D-Pen^2,L-Pen^5]enkephalin) and [2-D-penicillamine, 5-D-penicillamine]enkephalin ([D-Pen^2,D-Pen^5]enkephalin) were synthesized by solid-phase methods. Selectivities of these analogs for a single class of opioid receptor were investigated by examining relative potencies in the mouse vas deferens assay, in which the functional receptor is the δ receptor, versus the guinea pig ileum assay, in which the μ receptor is the functional receptor, and by determining their relative abilities to displace the prototypical δ receptor ligand [D-Ala^2,D-Leu^5]enkephalin and the prototypical μ receptor ligand naloxone from rat brain membrane preparations. Based on these comparisons [D-Pen^2,L-Pen^5]- and [D-Pen^2,D-Pen^5]enkephalin exhibited δ receptor selectivities of 1,088 and 3,164, respectively, in the bioassays, and 371 and 175, respectively, in the binding assays. Compared with the previously reported δ receptor selective analogs, [D-Ala^2,D-Leu^5]enkephalin, [D-Ser^2,Leu^5,Thr^6]enkephalin, and [D-Thr^2,Leu^5,Thr^6]enkephalin, the bis-Pen-containing analogs provide an order of magnitude increase in δ receptor selectivity.**

The endogenous opioid pentapeptides [Met^5]enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and [Leu^5]enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) have been shown to interact with several classes of opioid receptors (1–3) that may mediate different physiological responses. Elucidation of the roles of the individual receptor classes has been hampered by the general lack of enkephalin analogs with a high degree of selectivity for a single receptor type. The vast majority of analogs crossreact extensively with the different receptors, making it difficult to define receptor roles. This situation has been in part ameliorated by recent reports of an enkephalin analog highly selective for the μ opioid receptor (4–6) and a nonpeptide opiate with high κ receptor selectivity (7). However, analogs with corresponding selectivity for the δ opioid receptor have not been demonstrated.

One approach for the design of more selective analogs involves the incorporation of conformational restrictions. The native enkephalins, like most small, linear peptides, possess considerable conformational flexibility and by virtue of this flexibility can attain the presumably different conformational features required for interaction with different classes of opioid receptors. In principle, appropriate restriction of this flexibility can lead to analogs able to assume the conformation required to interact favorably with only one class of receptor. One method for effecting conformational restrictions is via cyclization of the peptide that constrains the resulting analog to assume a compact topography. Several active, cyclic enkephalin analogs have been reported, all of which are cyclized by either side chain to carboxyl terminus (8, 9) or side chain to side chain linkages (10–12). Of particular interest in the latter category is the finding that [D-Cys^2,L-Cys^5]- and [D-Cys^2,D-Cys^5]enkephalinamide, in which cyclization is achieved by means of a disulfide linkage connecting the two cysteine residues, display moderate μ receptor selectivities (10), whereas the cyclic analogs [D-Pen^2,L-Cys^5]- and [D-Pen^2,D-Cys^5]enkephalinamide (where Pen is penicillamine), which differ from the two former compounds only by virtue of the substitution of cysteine by Pen (β,β-dimethyl-cysteine) at the second residue, exhibit substantial δ receptor selectivities (11). The corresponding carboxylic acid terminal analogs [D-Pen^2,L-Cys^5]- and [D-Pen^2,D-Cys^5]enkephalin display even more pronounced δ receptor selectivities (12).

It has previously been shown that, in aqueous solution, the tocin ring portion of Pen-containing oxytocin analogs is conformationally restricted, whereas the tocin ring of oxytocin itself is quite flexible (13–16). This difference arises from the rigidifying effect of *gem*-dialkyl substituents in medium-sized rings and suggests that the δ opioid receptor selectivities observed for the [D-Pen^2,L(or D)Cys^5]enkephalins and enkephalinamides are also due to increased conformational rigidity. To investigate the effect of further rigidification, the bis-Pen-containing cyclic enkephalin analogs H-Tyr-D-Pen-Gly-Phe-L-Pen-OH ([D-Pen^2,L-Pen^5]enkephalin) and H-Tyr-D-Pen-Gly-Phe-D-Pen-OH ([D-Pen^2,D-Pen^5]enkephalin) (Fig. 1) were synthesized. Opioid receptor selectivity was evaluated in the mouse vas deferens (MVD) and guinea pig ileum (GPI) assays and in rat brain membrane binding assays. The bis-Pen-containing peptides were compared with the previously reported δ receptor selective analogs [D-Ser^2,Leu^5,Thr^6]enkephalin (17), [D-Thr^2,Leu^5,Thr^6]enkephalin (18), and [D-Ala^2,D-Leu^5]enkephalin (19).

MATERIALS AND METHODS

[D-Ala^2,D-Leu^5]Enkephalin was purchased from Sigma. The analogs [D-Pen^2,L-Pen^5]enkephalin, [D-Pen^2,D-Pen^5]enkephalin, [D-Ser^2,Leu^5,Thr^6]enkephalin (17), and [D-Thr^2,Leu^5,-Thr^6]enkephalin (18) were synthesized by solid-phase methods similar to those previously reported (11, 12). Chloromethylated (1.16 mmol/g of resin) polystyrene resin 1% crosslinked with

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Abbreviations: Standard abbreviations are used for the common amino acids, which, unless otherwise noted, are of the L-configuration. Other abbreviations are: Pen, penicillamine; MVD, mouse vas deferens; GPI, guinea pig ileum.

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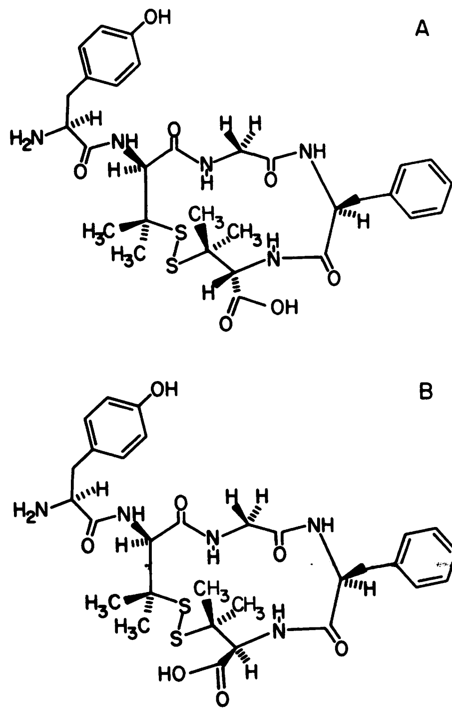

FIG. 1. bis-Pen cyclic enkephalin analogs [D-Pen^2,L-Pen^5]enkephalin (*A*) and [D-Pen^2,D-Pen^5]enkephalin (*B*).

divinylbenzene (Lab Systems, San Mateo, CA) was used as the solid support. N^{α}-*tert*-Butyloxycarbonyl-protected amino acids were used throughout. Benzyl protection of the threonine and serine side chains and *p*-methylbenzyl protection of the Pen side chains were utilized. Cleavage from the resin and removal of side chain protection were effected by treatment with anhydrous HF (10 ml/g of resin) in the presence of anisole (2 ml/g of resin) for 60 min at 0°C. For the sulfur-containing analogs, dithioethane (0.2 ml/g of resin) was also present. The peptides were extracted from the HF-treated resin with N_2-purged glacial acetic acid and the resin was then washed sequentially with 30% acetic acid, 0.2 M acetic acid, and water, all of which had been purged with N_2. The filtrates were combined and lyophilized. The product was dissolved in N_2-purged 0.1% acetic acid (*ca.* 0.25 mmol of peptide per liter of solution), the pH was adjusted to 8.5 with 4 M NH_4OH, and the disulfide was formed via oxidation by 0.01 M $K_3Fe(CN)_6$ (2-fold excess) for 2–4 hr. After the oxidation, the pH was adjusted to 4 with acetic acid and 10 ml of settled volume of AG-3X4A anion exchange resin (chloride form, Bio-Rad) was added. The mixture was stirred for 20 min and filtered, and the resin was washed three times with 50 ml each of 30% acetic acid. The volume of the filtrate was reduced by rotary evaporation and then lyophilized. The peptides were purified by partition chromatography on Sephadex G-25 block polymerizate by using the solvent system 1-butanol/acetic acid/water, 4:1:5 (vol/vol), followed by gel filtration on Sephadex G-15 with 30% acetic acid as the eluant. Purity of the enkephalin analogs was assessed by TLC, each compound yielding a single uniform spot in the four solvent systems: (*i*) 1-butanol/acetic acid/water, 4:1:5 (vol/vol), upper phase only; (*ii*) 1-butanol/pyridine/acetic acid/water, 15:10:3:12 (vol/vol); (*iii*) 1-butanol/water (3.5% acetic acid/1.5% pyridine), 1:1 (vol/vol), upper phase only; and (*iv*) 1-amyl alcohol/pyridine/water, 7:7:6 (vol/vol), and by HPLC utilizing a Waters RCM-100 Radical Compression Unit (C_{18} RP column, 8-mm inside diameter, 10-μm particle size) with isochratic elution by the solvent system: 1% trifluoroacetic acid (aqueous)/acetonitrile, 76:24 (vol/vol), at a flow rate of 2 ml/min with optical detection at 280 nm. The HPLC chromatograms indicated purities in excess of 98%. Amino acid analyses were performed on a Beckman 120C amino acid analyzer.

[D-Pen^2,L-Pen^5]Enkephalin. TLC R_f (solvent system in parenthesis): 0.69 (*i*); 0.83 (*ii*); 0.32 (*iii*); 0.70 (*iv*). HPLC k' (capacity factor): 2.54. Amino acid analysis: Tyr, 1.01; Gly, 0.97; Phe, 1.04; Pen, not determined.

[D-Pen^2,D-Pen^5]Enkephalin. TLC R_f (solvent system in parenthesis): 0.64 (*i*); 0.82 (*ii*); 0.32 (*iii*); 0.72 (*iv*). HPLC k' (capacity factor ratio): 2.54. Amino acid analysis: Tyr, 1.05; Gly, 0.96; Phe, 1.00; Pen, not determined.

GPI and MVD Bioassays. Assays based on the inhibition of electrically induced smooth muscle contractions were patterned after the standard procedures for the GPI longitudinal muscle-myenteric plexus preparation (20) and the isolated MVD (21) system and were performed as reported (11). Reported IC_{50} values represent the mean (± SEM) of three or four determinations. K_e (equilibrium constant) values of the antagonist naloxone were calculated by using the formula $K_e = Q/(DR - 1)$, in which Q is the antagonist (naloxone) concentration (nM) and DR is the dose ratio in naloxone-treated versus control preparations.

Receptor Binding Assays. Specific ligand binding studies using twice-washed rat brain (less cerebellum) membrane preparations [100 μl of 8% (wt/vol) homogenate] were performed as described (11). Concentrations of radioligands were 1 nM. Nonspecific binding was defined by 1 μM [Met]enkephalin or by 1 μM naltrexone in [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin or [^{3}H]naloxone binding assays, respectively. Incubations were performed in sodium-free buffer (50 mM Tris·HCl, pH 7.4, at 25°C containing 100 μg of bacitracin per ml) for 60 min for [^{3}H]naloxone (42.3 Ci/mmol, 1 Ci = 3.7×10^{10} Bq; New England Nuclear) and for 40 min for [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin (39.5 Ci/mmol; New England Nuclear). Reported IC_{50} values (± SEM) represent the concentration of inhibitor that caused half-maximal inhibition of specific [^{3}H]naloxone or [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin binding. All assays were carried out in triplicate on three to six membrane preparations.

RESULTS AND DISCUSSION

As summarized in Table 1, all the analogs tested inhibited the electrically induced contractions of GPI and MVD preparations. These effects were naloxone reversible. All analogs were more potent in the MVD system, in which the functional receptor is believed to be the δ receptor (1, 19), than in the GPI system, in which the μ receptor is thought to mediate the effect (1, 19). The large difference in K_e values for naloxone (Table 1) in the two preparations supports the conclusion that the tested analogs act via different receptors in the MVD and in the GPI. The ratios of the IC_{50} values in the GPI assay to those in the MVD assay can then be taken as an index of the selectivity of each analog for the δ versus the μ receptor. Based on this ratio, [D-Ala^2,D-Leu^5]enkephalin, the heretofore prototypical δ receptor ligand, was the least selective analog tested, whereas [D-Pen^2,L-Pen^5]- and [D-Pen^2,D-Pen^5]enkephalin were the most selective, exhibiting IC_{50} ratios of 1,088 and 3,164, respectively. In the case of [D-Pen^2,D-Pen^5]enkephalin, this represents 9.5 and 18 times the δ receptor selectivity that we find for the recently reported δ receptor-selective analogs [D-Ser^2,Leu^5,Thr^6]enkephalin (17) and [D-Thr^2,Leu^5,Thr^6]enkephalin (18), respectively.

Similar trends were observed in binding assays using rat brain membrane preparations as shown in Table 2. All of the peptides tested displaced the ^{3}H-labeled compounds in a multiphasic

Table 1. Inhibitory potencies (IC_{50}) of enkephalin analogs in GPI and MVD assays

Analog	IC_{50}, nM		Ratio*	$K_{e(\text{Naloxone})}$, nM	
	GPI	MVD		GPI	MVD
[D-Pen^2,L-Pen^5]Enkephalin	2,720 ± 50.1	2.50 ± 0.03	1,088	1.1 ± 0.2	49.4 ± 1.9
[D-Pen^2,D-Pen^5]Enkephalin	6,930 ± 124	2.19 ± 0.30	3,164	2.7 ± 0.7	45.7 ± 9.1
[D-Ala^2,D-Leu^5]Enkephalin	24.3 ± 5.3	0.27 ± 0.06	90	2.1 ± 0.6	36.2 ± 11.1
[D-Ser^2,Leu^5,Thr^6]Enkephalin	234.4 ± 85.6	0.70 ± 0.08	333	1.0 ± 0.1	28.8 ± 1.2
[D-Thr^2,Leu^5,Thr^6]Enkephalin	100.2 ± 19.6	0.58 ± 0.06	173	1.8 ± 0.2	52.4 ± 10.4
Normorphine	91 ± 19	540 ± 113	0.17	2.5 ± 0.4	4.3 ± 0.5

Reported IC_{50} values represent the mean (± SEM) of three or four determinations. K_e was calculated by using the formula $K_e = Q/(DR - 1)$, in which Q = antagonist (naloxone) concentration and DR = dose ratio in naloxone-treated versus control preparations.
* GPI IC_{50}/MVD IC_{50}.

manner, with Hill slopes significantly less than 1. [D-Ala^2,D-Leu^5]Enkephalin was the least selective analog, exhibiting a 4-fold higher potency for displacement of [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin (δ receptor agonist) than for displacement of [^{3}H]naloxone (μ receptor antagonist). [D-Thr^2,Leu^5,Thr^6]-Enkephalin was marginally more selective than [D-Ala^2,D-Leu^5]-enkephalin, whereas [D-Ser^2,Leu^5,Thr^6]enkephalin provided a 4-fold improvement over [D-Ala^2,D-Leu^5]enkephalin (IC_{50} ratio of 15.4). By contrast, the bis-Pen-containing analogs showed very high selectivities in these assays with IC_{50} (naloxone)/IC_{50} ([D-Ala^2,D-Leu^5]enkephalin) ratios of 371 for [D-Pen^2,L-Pen^5]enkephalin and 175 for [D-Pen^2,D-Pen^5]enkephalin. Compared with [D-Ser^2,Leu^5,Thr^6]enkephalin, in our hands the most selective of the previously reported putative δ receptor ligands, [D-Pen^2,L-Pen^5]- and [D-Pen^2,D-Pen^5]enkephalin provided an improvement in specificity of 24- and 11-fold, respectively. The δ selectivities of the bis-Pen enkephalin analogs are also substantially higher than those reported for the dimeric enkephalin analog (H-Tyr-D-Ala-Gly-Phe-NH-)$_2$-(-CH_2-)$_{12}$, for which receptor selectivity was assessed by comparing binding to δ receptors of neuroblastoma × glioma membranes with binding to μ sites in rat brain preparations (22). The low selectivity of [D-Ala^2,D-Leu^5]enkephalin observed in our results and reported by others (6) and the previously described crossreactivity of naloxone with μ and δ sites (23) indicate that the IC_{50} ratios given in Table 2 are not rigorous measures of δ versus μ receptor selectivities. Nonetheless, these ratios are valid for assessing the relative selectivities of the tested analogs.

The extraordinarily high δ receptor selectivities of the cyclic, bis-Pen enkephalin analogs represent an order of magnitude improvement over previously reported δ selective analogs, which indicates that these compounds will prove valuable for determining the physiological role of this receptor. Further, the conformationally restricted nature of these analogs renders them amenable to conformational analysis in solution because they are less subject to dynamic averaging than are more flexible compounds. More importantly, the conformational restrictions imposed in a 14-membered ring system by the bis-Pen substitution require that the three-dimensional structures of these analogs when bound to the receptor must closely approximate those observed in solution. Thus, careful conformational analysis of the bis-Pen analogs in aqueous solution should provide important insights into the conformational requirements for δ receptor–ligand binding and transduction. Such extrapolations from solution to receptor-bound conformation for more flexible analogs generally are not valid. Investigations into the solution conformation of the enkephalins and their linear analogs have led to the proposal of several mutually incompatible models (24–26). Conformational analysis of more rigid enkephalin analogs such as the Pen-containing compounds of this and previous reports (11, 12) will allow these discrepancies to be resolved.

It recently has been proposed that favorable interaction with μ receptors requires a compact comformation of the ligand, whereas favorable interaction with the δ receptor requires a more extended conformation of the ligand (27). The observed μ receptor selectivities of cyclic enkephalin analogs prepared by Schiller and co-workers (8–10) are in agreement with the former proposal. However, the very high δ receptor selectivities of the bis-Pen analogs presented here and of previously reported Pen-containing enkephalin analogs (11, 12) cast serious doubt on the latter suggestion. Rather, the results reported here indicate that the conformational requirements for optimal interaction with the μ and δ receptors differ in a more subtle manner. Biophysical studies using these conformationally re-

Table 2. Receptor binding affinities of enkephalin analogs for [^{3}H]naloxone and [D-Ala^2,D-Leu^5]-[^{3}H]enkephalin sites in rat brain homogenates

Analog	IC_{50}, nM		Ratio*
	[^{3}H]Naloxone	[D-Ala^2,D-Leu^5]-[^{3}H]Enkephalin	
[D-Pen^2,L-Pen^5]Enkephalin	3,710 ± 740	10.0 ± 0.2	371
[D-Pen^2,D-Pen^5]Enkephalin	2,840 ± 670	16.2 ± 0.9	175
[D-Ala^2,D-Leu^5]Enkephalin	16 ± 5.0	3.9 ± 0.7	4.1
[D-Ser^2,Leu^5,Thr^6]Enkephalin	88 ± 6.0	5.7 ± 0.4	15.4
[D-Thr^2,Leu^5,Thr^6]Enkephalin	36.3 ± 3.8	6.4 ± 0.6	5.7
Morphine	23.3 ± 2.4	27.2 ± 1.2	0.9

IC_{50} values represent the mean concentration (± SEM) of analog that caused half-maximal inhibition of specific ^{3}H-labeled ligand. All incubations were carried out at 25°C in 50 mM Tris·HCl buffer (pH 7.4) by using twice-washed membrane homogenates of whole rat brain minus cerebellum.
* Naloxone IC_{50}/[D-Ala^2,D-Leu^5]enkephalin IC_{50}.

stricted analogs should provide important insights into these requirements. In addition, the δ selective analogs reported here should provide an important tool for determining the physiological role(s) of the δ opioid receptor.

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