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Structural Requirements for δ Opioid Receptor Binding

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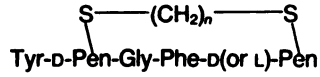
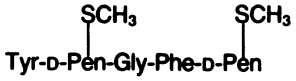
SUMMARY

Structural features influencing opioid activity of enkephalin analogs were investigated through the synthesis and evaluation of opioid receptor binding affinities of a series of cyclic dithioether-containing analogs and structurally related linear analogs of the cyclic, disulfide-containing peptides, [D-Pen^2, D-Pen^5]enkephalin and [D-Pen^2, L-Pen^5]enkephalin, where Pen (penicillamine) is β,β-dimethylcysteine. The major effect of increasing the ring size of the cyclic moiety from disulfide to dithioether analogs was a large decrease in δ opioid receptor binding affinity which suggests that relatively compact conformations of the peptide ligand are necessary for optimal binding to this receptor. The effect of bulky, hydrophobic residues at position 2 in the peptide chain was evaluated by preparing the linear analogs, [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin (*t*-Leu, 2-amino-3,3-dimethylbutanoic acid) and [D-Abu^2, D-*t*-Leu^5]enkephalin (Abu, 2-aminobutanoic acid). The former analog was found to be 36- and 450-fold less potent at δ and μ receptor sites, respectively, than was the latter, suggesting that bulky side chain substituents in position 2 of enkephalin analogs lead to a deleterious steric interaction at δ and particularly at μ receptors.

The cyclic enkephalin analogs, DPDPE and DPLPE, where Pen (penicillamine) is β,β-dimethylcysteine, have been shown to display high selectivity for the δ type of opioid receptor (1–3). These bis-penicillamine-containing enkephalins are conformationally restricted because of the imposed cyclization through the side chain sulfurs via a disulfide linkage and are further restricted because of the rigidizing effect of the penicillamine *gem* dimethyl groups (1). Although DPDPE and DPLPE are potent agonists at the δ receptor, a potential disadvantage of such analogs in general is that the conformational restriction imposed may be too severe to allow optimal interaction with the desired receptor. We previously proposed an approach to examine this possibility by the synthesis of dithioether-containing cyclic analogs through which the effect of ring size and, thus, conformation on pharmacological activity can be assessed (4). We report here an application of this approach to the synthesis and opioid receptor binding evaluation of a series of analogs related to DPDPE and DPLPE of the general structure:



where n = 1, 2, or 3 for the dithioether-containing analogs and n = 0 for DPDPE and DPLPE. We also report the synthesis and binding affinities of the related, linear thioether-containing analog:



as well as the linear analogs [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin, which is isosteric with DPDPE(SH)$_2$, the linear, free sulfhydryl-containing synthetic precursor of DPDPE, and [D-Abu^2, D-*t*-Leu^5]enkephalin, in which Abu is 2-aminobutanoic acid and *t*-Leu, *tert*-leucine, is 2-amino-3,3-dimethylbutanoic acid. For ease of notation, we designate the dithioether-containing analogs by indicating the parent disulfide-containing analog (DPDPE or DPLPE) followed in parentheses by the alkyl group inserted between the sulfurs. Thus, for example, DPDPE(CH$_2$)$_2$ indicates the ethylene dithioether analog of DPDPE. By extension of this notation, the linear thioether analog is abbreviated as DPDPE(CH$_3$)$_2$ to indicate the presence of the methyl thioethers. These analogs provide a means of assessing the degree of folding required for optimal interaction with the δ opioid receptor-binding site and provide a test of the hypothesis proposed by Roques and co-workers (5) that a relatively extended conformation of enkephalin analogs yields optimal binding to δ receptors.

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ABBREVIATIONS: DPDPE, [D-Pen^2, D-Pen^5]enkephalin; DPLPE, [D-Pen^2, L-Pen^5]enkephalin; Pen, penicillamine; *t*-Leu, *tert*-leucine, 2-amino-3,3-dimethylbutanoic acid; Abu, 2-aminobutanoic acid; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; DAGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; BREM, bremazocine; U50,488, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide; RSI, receptor selectivity index.

Materials and Methods

Peptide synthesis. DPDPE(SH)$_2$ and DPLPE(SH)$_2$, the free sulfhydryl-containing, linear precursors of DPDPE and DPLPE, respectively, were prepared using solid phase methods followed by cleavage from the solid support by treatment with anhydrous HF containing 10% anisole as previously described (1). The peptides were purified by HPLC on a Vydac 218TP C-18 column (2.5 × 22 cm) under isocratic conditions using the solvent system 0.1% TFA in H_2O/0.1% TFA in acetonitrile (75/25). Dithioether-containing, cyclic analogs were prepared from the appropriate purified, sulfhydryl-containing peptides using a modification of our previously reported procedure (4). DPDPE(SH)$_2$ or DPLPE(SH)$_2$ was dissolved in nitrogen-purged dimethylformamide (0.1 mg/ml). After the addition of a 5.5-fold molar excess of potassium-*tert*-butoxide, a 1.5-fold molar excess of dibromomethane, 1,2-dibromoethane, or 1,3-dibromopropane in 10 ml of dimethylformamide was added dropwise, with stirring, over a period of 1 hr. The reaction was then quenched by the addition of glacial acetic acid and the solvent was removed by rotary evaporation. The product dithioether-containing peptides were purified by HPLC as above using the solvent system 0.1% TFA in H_2O/0.1% TFA in acetonitrile (72/28). Purity of the product peptides were assessed by analytical HPLC utilizing a linear gradient of solvents described above. All peptides were greater than 98% pure by this measure. The purified putative dithioether-containing enkephalins were tested for the presence of free sulfhydryl groups by the use of 5,5′-dithiobis-(2-nitrobenzoic acid) (6) with and without prior incubation with disulfide reducing agents (2-mercaptoethanol, dithiothreitol, or $NaBH_4$). In all cases these tests were negative, indicating the absence of free sulfhydryls or disulfides. Analysis by fast atom bombardment mass spectrometry yielded the appropriate molecular weights for these peptides. Proton NMR spectra of all peptides were consistent with the proposed structures.

Radioreceptor binding assays. A freshly removed guinea pig brain was weighed (about 3 g), frozen immediately on dry ice, and homogenized (Tekmar Tissumizer, Cincinnati, OH) in 30 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, bubbled continuously with CO_2 at 37°. After centrifuging (12,000 × *g*, 23°, 10 min), the pellet was resuspended in the same buffer to about 90 ml, then incubated at 37° for 20 min in tightly capped tubes to destroy endogenous opioids. After centrifuging again, the membranes were resuspended to about 180 ml, subdivided into three portions, each made up to 200 ml, capped again, and incubated as above. The membranes were then washed by two rounds of centrifuging (16,000 × *g*, 23°, 10 min) and resuspended in Tris buffer (50 mM, pH 7.4). The membrane pellet was dispersed with a Dounce B homogenizer at 23° to make a homogenous suspension (typically 150 ml) representing 20 mg of original brain per ml of buffer, with a protein content of 0.2 mg/ml.

For the μ system we used 0.8 nM ^{3}H-labeled DAGO, (New England Nuclear, Boston, MA, 48 Ci/mmol), with 300 nM unlabeled DAGO as total displacement competing ligand to define nonspecific binding. For the δ system we used 1.0 nM [^{3}H]DPDPE (Amersham, Chicago, IL, 28 Ci/mmol) with 100 nM DPDPE as the total displacement competing ligand. Specific binding was 83% and 70% of total binding for [^{3}H]DAGO and [^{3}H]DPDPE, respectively.

For κ sites no sufficiently selective radioligand was available, therefore, a "paired-tube" paradigm was devised. [^{3}H]BREM (New England Nuclear, 30 Ci/mmol) is a high affinity radioligand with only modest selectivity for κ sites, but U50,488 (Upjohn, Kalamazoo, MI), not available as a radioligand, is highly selective for κ sites. Specific κ binding was therefore defined as the difference between the binding of 75 pM [^{3}H]BREM in the absence and presence of U50,488. We used [^{3}H]BREM with and without 100 nM U50,488 in matched pairs of tubes at every concentration of a competing ligand. In these experiments 5 nM DAGO and 50 nM DPDPE were also present to block μ and δ sites, but the paired-tube differences proved to be virtually the same with and without the blockers. Specific κ binding was typically about 88% of total binding.

The incubation mixture contained 1.0 ml of the membrane suspension and 1.0 ml of Tris buffer containing radioligand and blocking and displacing ligands as required. To this was added 10 μl of methanol/0.1 M HCl (1:1, v/v) containing competing ligand, or methanol/HCl alone (which had no effect on the assays). Incubation was at 23° for 2 hr, demonstrated with each radioligand and competing ligand to be long enough for equilibration. Incubation was terminated by chilling on ice, then adding 4 ml of ice-cold buffer and collecting on filters (No. 32 S&S glass fiber strips presoaked in glass-distilled water saturated with *t*-amyl alcohol) in a cell harvester (M24R, Brandel, Gaithersburg, MD). Tubes were washed onto the filters three times with 4 ml of cold buffer. Filters were transferred to scintillation vials and allowed to stand at room temperature in 5 ml of Cytoscint for at least 5 hr; then, radioactivity was counted to 1.5% SD.

Free radioligand concentrations (used in all calculations) were measured by determining the radioactivity of supernatants from parallel sets of tubes centrifuged (instead of filtered) after the incubation. The free concentration when a competing ligand reduced bound radioligand by half (see below), was estimated by interpolation. Radioligand K_d values were determined from computer fits to the high affinity portion of binding isotherms.

Radioligand depletion by binding must be taken into account if it is greater than a few per cent (7–9). At the radioligand concentrations used, the percentage of depletion by binding in the absence of a competing ligand was 6 ± 2% (SE) of [^{3}H]DAGO (N = 3), 8 ± 2% of [^{3}H]DPDPE (N = 11), and 43 ± 1% of [^{3}H]BREM (N = 17). Accordingly, a rigorous equation derived from the law of mass action was used instead of the Cheng-Prusoff approximation (10) to convert IC_{50} to K_i for each competing ligand,

$$K_i = \{ 1/[(2L/L_o) + (L/K_d) - 1] \} IC_{50}$$

where the measured free radioligand concentrations are L_o in the absence of a competing ligand and L at the IC_{50}.

Radioligands were checked periodically for purity by reverse phase HPLC, and were purified by preparative HPLC if necessary. They were also demonstrated, by the same method, to be undegraded after the 2-hr incubation with membranes.

That the three binding procedures described here are highly selective for μ, δ, and κ sites is indicated by the smoothness, symmetry, and slopes of all competition curves (not shown). The RSIs of the key ligands DAGO, DPDPE, and U50,488 (see Table 1) permit one to compute the distribution of each radioligand between its primary (preferred) site and its secondary (next-preferred) site. Even assuming the unfavorable case that there are 3 times as many secondary as primary sites, only 0.5% of [^{3}H]DAGO or 1.3% of [^{3}H]DPDPE would label δ and μ sites, respectively, and less than 1% of the [^{3}H]BREM displacement by U50,488 would be at sites other than κ.

Results and Discussion

Binding affinities of the compounds under study at μ, δ, and κ opioid receptors in guinea pig brain are summarized in Table 1 as pK_i values. Also presented in Table 1 are the RSIs of each compound where RSI is the ratio of the K_i at a given site to the K_i at the highest affinity site. As shown in Table 1 and as previously observed (1–3), DPDPE and DPLPE bind with high affinity to δ opioid receptors, whereas their affinities for μ receptors are 260- and 300-fold lower, respectively. DPDPE and DPLPE and, indeed, all of the analogs listed in Table 1, with the exception of U50,488, have very weak affinities for κ receptors.

For the homologous series of cyclic, dithioether-containing analogs, the results in Table 1 show that μ receptor binding affinities display only modest changes relative to the corresponding cyclic, disulfide-containing analog. Interestingly, increasing the ring size from the 14-membered ring of the disulfide to the 15-membered ring of the methylene dithioether

TABLE 1

Opioid receptor binding selectivity profiles for enkephalin analogs

For each ligand and each type of binding site, three independent experiments were carried out. Concentration doublings were used to generate complete competition curves for the key ligands DAGO, DPDPE, and U50,488. IC_{50} values of test ligands were obtained by bracketing, with 10-fold concentration increments. K_i values were computed as described under Materials and Methods. All competition curves were smooth and symmetrical, and all log-logit slopes were compatible, within experimental error, with the value 1.0. Data in the table under pK_i are mean values of the negative logarithms of the computed K_i values. SE for independent triplicate determinations never exceeded 0.1 log unit. RSI is the ratio of K_i at a secondary (nonpreferred) site to that at the primary (highest affinity) site. All but the last three ligands in the table and the reference compounds DAGO and U50,488 are cyclic (see the text).

Analog	Opioid-binding site					
	μ		δ		κ	
	pK_i	RSI	pK_i	RSI	pK_i	RSI
DAGO	9.59		6.73	720	6.01	3,800
U50,488	6.72	260	5.11	10,000	9.13	
DPDPE	6.61	260	9.02		4.92	13,000
DPLPE	6.78	300	9.25		<4.63	>42,000
DPDPE (CH_2)	7.15	1.4	7.31		<4.63	>480
DPLPE(CH_2)	7.57	6.9	8.41		<4.63	>6,000
DPDPE(CH_2)$_2$	6.28	16	7.49		<4.63	>720
DPLPE(CH_2)$_2$	6.99	2.2	7.33		<4.63	>500
DPDPE(CH_2)$_3$	6.29	4.1	6.90		<4.63	>190
DPLPE(CH_2)$_3$	6.41	15	7.58		<4.63	>890
DPDPE(CH_3)$_2$	6.80	2.3	7.16		4.69	300
[D-Abu^2, D-*t*-Leu^5]Enkephalin	8.65		7.83	6.6	4.70	8,900
[D-*t*-Leu^2, D-*t*-Leu^5]Enkephalin	6.00	1.9	6.27		4.83	28

(actually a dithioacetal) results in a slight increase in μ receptor binding affinity [3.5-fold for DPDPE(CH_2) versus DPDPE; 6-fold for DPLPE(CH_2) versus DPLPE]. The further increases in ring size afforded by the ethylene and propylene dithioethers lead to decreased μ receptor binding affinities with values similar to those for the corresponding disulfide-containing analogs.

A considerably different effect of ring size on δ receptor binding affinities is apparent from Table 1. For the DPDPE-related series, DPDPE(CH_2) and DPDPE(CH_2)$_2$ display similar binding affinities which are ~50-fold lower than that of DPDPE. Further increase in ring size to DPDPE(CH_2)$_3$ results in an additional 4-fold reduction in binding affinity. Compared with DPLPE, DPLPE(CH_2) also shows reduced δ receptor binding affinity but is still fairly potent. Further increase in ring size to DPLPE(CH_2)$_2$ and DPLPE(CH_2)$_3$ reduces potency by an additional factor of ~10.

The binding affinities of the linear analogs, DPDPE(CH_3)$_2$, [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin, and [D-Abu^2, D-*t*-Leu^5]enkephalin are also shown in Table 1. DPDPE(CH_3)$_2$, which is isosteric with the cyclic dithioether analog, DPDPE(CH_2)$_2$, but is not constrained by cyclization, is approximately 3-fold more potent at μ receptor binding sites and 2-fold less potent at δ receptor binding sites than is DPDPE(CH_2)$_2$. Although these differences are rather small, they are consistent with the general trend which emerges from the dithioether series, namely, that more compact structures (such as in DPDPE and DPLPE) favor δ receptor binding. The binding results for [D-*t*-Leu^2, D-*t*-Leu^5] enkephalin and [D-Abu^2, D-*t*-Leu^5]enkephalin provide further insights. [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin, which is sterically similar to the free sulfhydryl-containing, linear precursor of DPDPE, displays the lowest binding affinity at μ and δ receptors of any analog listed in Table 1. Compared with DPDPE, [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin is 4-fold less potent at μ and 560-fold less potent at δ receptor binding sites. From the known van der Waals radii of the side chain atoms it can be shown that [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin cannot adopt as compact a structure as DPDPE, suggesting that at least part of the reduced δ receptor binding affinity of this linear analog is due to its more extended conformation. However, the reduced δ affinity of [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin relative to the dithioethers and the linear DPDPE(CH_3)$_2$ further suggests that the electronegative side chain sulfurs may play a role in the δ receptor binding interactions of the latter analogs and particularly of DPDPE and DPLPE. These sulfur atoms may provide a portion of the binding energy by functioning as hydrogen bond acceptors, a function that the corresponding methyl group of D-*t*-Leu cannot fulfill. The relative importance of conformation versus hydrogen bonding capability could be assessed by determining the δ receptor binding affinity of DPDPE(SH)$_2$; however, this free sulfhydryl-containing, linear precursor of DPDPE could not be tested because it was found to be converted to DPDPE during the incubation. Attempts to assess the binding of DPDPE(SH)$_2$ in the presence of the reducing agent dithiothreitol were also unsuccessful, it being found that concentrations of dithiothreitol sufficient to prevent conversion of DPDPE(SH)$_2$ to DPDPE interfered with the binding assay.

The influence of steric effects in position 2 of enkephalin analogs on opioid receptor binding affinity can also be deduced from Table 1 by comparing the results for [D-*t*-Leu^2, D-*t*-Leu^5] enkephalin with those for [D-Abu^2, D-*t*-Leu^5]enkephalin. The latter analog which lacks the former's β,β-dimethyl substituent in the second residue is 36-fold more potent at δ receptor binding sites and 450-fold more potent at μ receptor binding sites. As a result, [D-Abu^2, D-*t*-Leu^5]enkephalin exhibits modest μ receptor binding selectivity. This result suggests that the β,β-dimethyl substituents in [D-*t*-Leu^2, D-*t*-Leu^5]enkephalin and, by extension, in DPDPE reduce binding affinity at both μ and δ receptors, with a greater effect at μ receptors. This is consistent with the observation (11) that, while the conformations of DPDPE and the related cyclic analog [D-Cys^2, D-Pen^5]enkephalin, which lacks the β,β-dimethyl substituents at residue 2, are quite similar, the latter analog is considerably more potent and less δ receptor selective than is the former. It should be mentioned that previous reports have noted that bulky hydrophobic substituents at position 2 in enkephalin analogs lead to losses

in opioid potency (12, 13), and it has been proposed that hydrophilic residues at this position enhance δ receptor potency (5). The results presented here agree with these findings and further suggest that bulky substituents at position 2 are more deleterious toward μ receptor binding than toward δ receptor binding. Furthermore, the results presented here are at odds with the hypothesis that relatively extended conformations of enkephalin analogs favor δ opioid receptor activity (5). Rather, they suggest that compact, folded conformations are necessary for optimal interaction with δ opioid receptors. The ability of DPDPE and DPLPE to assume such compact conformations is thus responsible for the outstanding δ receptor binding selectivities and potencies displayed by these ligands.

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