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Dimeric Enkephalins Display Enhanced Affinity and Selectivity for the *Delta* Opiate Receptor

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SUMMARY

We have synthesized a series of dimeric analogues of [D-Ala^2,Leu^5]enkephalin, cross-linked at the COOH terminus by α,ω-diaminoalkanes of variable length. The structure of the purified peptides has been confirmed by amino acid analysis and by molecular weight determination using mass spectrometry. The peptides were evaluated for activity in three radioligand assay systems, employing membranes from rat brain, using [^{3}H]naloxone, ^{125}I-[D-Ala^2,D-Leu^5]enkephalin, and ^{3}H-[D-Ala^2,Met^5]enkephalin amide, to evaluate activity for the "μ" and "δ" opiate receptors. The dimeric pentapeptide enkephalin analogues were less potent than the monomeric analogue ([D-Ala^2,Leu^5]enkephalin amide) when [^{3}H]naloxone was used as the labeled ligand, but showed a striking increase in relative potency when ^{125}I-[D-Ala^2,D-Leu^5]enkephalin was utilized as tracer, indicating selectivity for the δ receptors. Intermediate values for relative potency were obtained with ^{3}H-[D-Ala^2,Met^5]enkephalin amide was utilized as tracer. Relative activity was highest when the cross-linking methylene bridge consisted of $n = 2$, 4, or 6 carbon atoms, and decreased dramatically for longer chain lengths ($n = 8$, 10, or 12). The selectivity of the compounds for the δ receptors appeared to be greatest when $n = 2$, and decreased progressively as the length of the methylene bridge was increased. These results indicate that dimeric pentapeptide enkephalins have increased affinity and selectivity for the δ receptor, and suggest the hypothesis that the dimeric enkephalins can cross-link opiate receptors. The peptides provide a new class of compounds for the study of opiate-receptor pharmacology.

INTRODUCTION

Elucidation of the properties of the "δ" enkephalin receptors (1–4) will require development of agonists and antagonists with higher affinity and selectivity than have heretofore been available. Unfortunately, the best enkephalin analogues, which are resistant to enzymatic hydrolysis and involve COOH-terminal modifications (5, 6), usually are associated with loss of δ versus "μ" selectivity, i.e., their pattern of activity resembles that of morphine rather than that of the naturally occurring peptides (3, 7). In the present study, we sought to increase the affinity by linking two enkephalin analogues together, thereby taking advantage of the increase in binding energy obtainable with multiple attachment to the receptor (8). We chose [D-Ala^2,Leu^5]enkephalin for dimerization because of its stability. The methylene bridge of varying length, $(\text{-CH}_2\text{-})_n$, with $n = 2$, 4, 6, 8, 10, and 12, was utilized to provide simplicity, flexibility, and chemical stability. The NH_2-terminal has clearly been established as essential for activity, whereas modifications of the COOH-terminal have relatively little effect on potency (9). Accordingly, we chose to cross-link the two enkephalin moieties through the COOH terminus. In the present report, we describe the synthesis of these DPE$_n$[1] and their physical properties, and provide evidence that they are active in opiate receptor systems, with very high affinity and δ specificity.

MATERIALS AND METHODS

Analytical Determinations

Melting points were measured utilizing a 6427-H10 Thomas-Hoover melting point apparatus and were uncorrected. TLC was carried out utilizing Silica Gel G (250 μm, Analtech). Optical rotations were measured with a Perkin-Elmer Model 241MC polarimeter. Mass spectra were obtained using Californium-252 plasma desorption mass spectrometry (10). Amino acid analyses were performed on a Beckman Model 121MB amino acid analyzer.

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[1] The abbreviations used are: DPE$_n$, dimeric pentapeptide enkephalins, where n denotes the length of the methylene chain; TLC, thin-layer chromatography; Boc, *tert*-butoxycarbonyl; DD, dimeric dipeptides; DMF, *N,N*-dimethylformamide; HOBt, 1-hydroxybenzotriazole; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; DP, dimeric pentapeptides; TFA, trifluoroacetic acid; NAL, naloxone; DAMEA, [D-Ala^2,Met^5]enkephalin amide; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DALEA, [D-Ala^2,Leu^5]enkephalin amide.

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TABLE 1

Physical properties of (Boc-Phe-Leu-NH)$_2$·(-CH$_2$-)$_n$ (DD$_n$)

DD$_n$	Yield	Melting point	$[\alpha]_D^{25}$ (*C* 1, DMF)	TLC[a]
	%			R_F
DD$_2$	84	213–216°	−13.7°	0.44
DD$_4$	96	186–188	−10.5	0.45
DD$_6$	73	178–180	−16.9	0.46
DD$_8$	75	148–149	−15.3	0.54
DD$_{10}$	96	131–134	−14.6	0.60
DD$_{12}$	79	120–121	−13.1	0.66

[a] Solvent: $CHCl_3$-EtOAc (1:1, v/v).

Peptide Synthesis

Step 1. (Boc-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$ (*n* = 2, 4, 6, 8, 10, and 12). The dimers of the Boc-Phe-Leu dipeptide are designated DD$_n$, where *n* denotes the length of the methylene chain. To a solution of Boc-Phe-Leu-OH (833 mg, 2.20 mmoles) and α,ω-diaminoalkanes, H_2N-(CH$_2$)$_n$-NH_2 (1.00 mmole) in DMF[1] (20 ml) were added HOBt (404 mg, 2.64 mmole) and EDC·HCl (464 mg, 2.42 mmole) at −10°. The reaction mixture was stirred for 2 hr at 0°, followed by incubation for 2 days at room temperature. The solvent was evaporated *in vacuo*, and ice-water was added to precipitate a solid. The solid collected was washed successively with 4% $NaHCO_3$, 10% citric acid, water, and petroleum ether. It was then dried, and recrystallized twice from DMF-EtOAc-ether. Analytical data are summarized in Table 1.

Step 2. (Boc-Tyr-D-Ala-Gly-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$ (*n* = 2–12). The dimers of the Boc-protected derivatives of the pentapeptide analogues are designated DP$_n$, where *n* again indicates the length of the cross-linking agent. Compound DD$_n$ (0.5 mmole) was dissolved in TFA (5 ml) at 0°. After 30 min at 0°, the solution was evaporated to leave an oil, which was solidified by the addition of anhydrous ether, yielding (TFA·H-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$. This TFA salt (0.5 mmole), Et_3N (0.14 ml, 1.0 mmole) and Boc-Tyr-D-Ala-Gly-OH (409 mg, 1.0 mmole) were dissolved in DMF (15 ml). To the solution were added HOBt (184 mg, 1.2 mmoles) and EDC·HCl (211 mg, 1.1 mmoles) at − 10°, and the reaction mixture was treated as described for the DD$_n$ series in Step 1. Purifications were carried out by gel filtration on a Sephadex LH-20 column (2.2 × 96 cm) eluted with DMF, and then by recrystallization from DMF-EtOAc-ether (Table 2).

Step 3. (AcOH·H-Tyr-D-Ala-Gly-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$ (*n* = 2–12). The liberated dimeric pentapeptide analogues are designated as DPE$_n$, where *n* again indicates the cross-linking chain length (Fig. 1). The compound DP$_n$ (0.25 mmole) was treated with TFA (5 ml) at 0° for 30 min. After evaporation of TFA the residual oil was dissolved in 30% AcOH and subjected to gel filtration on a column (2.2 × 145 cm) of Sephadex G-25 in 30% AcOH. The fractions containing a pure product by TLC were pooled and lyophilized repeatedly with aqueous AcOH. Homogeneity of the peptides was verified by ascending TLC in three different solvent systems (Table 3). For amino acid analysis, peptides were hydrolyzed in 6 M HCl for 24 hr at 110° in de-aerated tubes (Table 3).

Radioligand Binding Assays

Receptor binding assays using rat brain crude membrane preparations were performed essentially as described (11). Incubations were carried out for 1 hr at 25° in 50 mM Tris·HCl buffer (pH 7.5) containing bacitracin (100 μg/ml). ^{3}H-NAL (50.2 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) was used at a final concentration of 0.35 nM, and ^{3}H-DAMEA (40 Ci/mmole, New England Nuclear Corporation) at 0.25 nM. Monoiodinated ^{125}I-DADLE was obtained by the chloramine T iodination procedure (12), using a low ratio of $Na^{125}I$ to peptide, and was purified by high-performance liquid chromatography on a reversed-phase column using an MeOH-0.1 M NaH_2PO_4 solvent system. ^{125}I-DADLE was used at a final concentration of 0.03–0.05 nM. A total reaction volume of 2.0 ml was used for tritiated ligands, and 0.5–1.0 ml for the iodinated one. Rat brain membranes were used at a final dilution of 10 mg/ml (final wet weight). Peptide dimers were dissolved as stock solutions (1 mM) in 2% AcOH and stored in small aliquots at −20°. Immediately before use, serial dilutions of all peptides were prepared in 50 mM Tris·HCl buffer (pH 7.5) containing 0.1% ovalbumin. Dose-response curves were constructed utilizing eight or ten dose levels, in duplicate. All binding curves were repeated in at least three separate experiments. Results were analyzed by the computer program ALLFIT, to construct the least-

TABLE 2

Physical properties of (Boc-Tyr-D-Ala-Gly-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$ (DP$_n$)

DP$_n$	Yield	Melting point	$[\alpha]_D^{25}$ (*C* 1, DMF)	TLC[a]
	%			R_F
DP$_2$	83	204–205°	−11.7°	0.20
DP$_4$	82	166–168	−10.5	0.23
DP$_6$	94	209–211	−12.1	0.28
DP$_8$	89	210–213	−12.3	0.32
DP$_{10}$	88	200–202	−12.5	0.34
DP$_{12}$	81	165–166	−12.4	0.35

[a] Solvent: $CHCl_3$-MeOH (9:1, v/v).

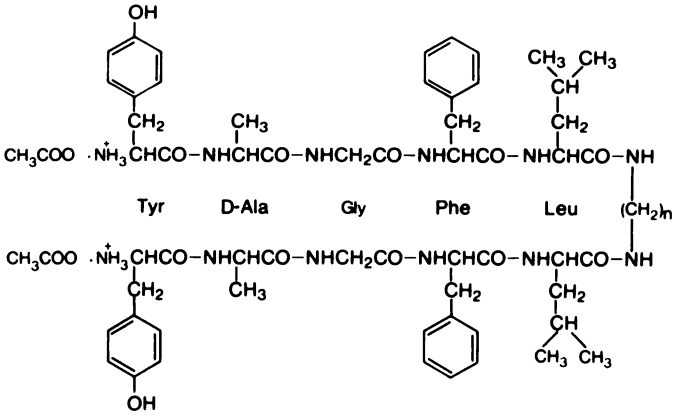

FIG. 1. *Structure of DPE$_n$*

TABLE 3

Physical properties of (AcOH·H-Tyr-D-Ala-Gly-Phe-Leu-NH-)$_2$·(-CH$_2$-)$_n$, (DPE$_n$)

DPE$_n$	Yield	Melting point	$[\alpha]_D^{20}$ (*C* 0.5, 95% AcOH)	TLC[a]	Mol wt[b] (M + Na$^+$)		Amino acid analysis				
					Found	Calculated	Tyr	Ala	Gly	Phe	Leu
	%			R_F							
DPE$_2$	98	126°	+22.6°	0.28	1185.79	1186.00	1.96	2.00	2.04	2.04	2.00
DPE$_4$	98	149	+21.0	0.29	1214.12	1214.44	1.96	2.00	2.02	2.04	2.00
DPE$_6$	96	118	+22.6	0.32	1242.18	1242.52	1.96	2.00	2.00	2.06	2.02
DPE$_8$	87	111	+23.4	0.35	1270.07	1270.54	1.96	2.02	2.04	2.10	2.04
DPE$_{10}$	96	109	+22.8	0.36	1298.24	1297.90	2.00	1.96	2.04	2.08	2.02
DPE$_{12}$	98	108	+22.2	0.38	1326.16	1326.65	1.94	2.00	1.98	2.08	2.02

[a] Solvent: *n*-BuOH-AcOH-H$_2$O (4:1:5, v/v, organic phase). R_F data were also obtained with the following solvent systems: 0.1% AcOH-*n*-BuOH-pyridine (11:5:3, v/v, organic phase), and *n*-BuOH-AcOH-pyridine-H$_2$O (15:3:10:12, v/v).

[b] Observed as salt-free peptides.

squares estimates of the logistic curves relating binding of labeled ligand to concentrations of unlabeled ligand (13). ED$_{50}$ values were obtained, together with their standard errors within experiments, and the standard error between experiments was calculated on the basis of a log-normal distribution.

RESULTS

Peptide synthesis was performed by a conventional solution method (Fig. 2; Tables 1–3). The dimeric pentapeptide enkephalin analogues (DPE$_n$) were proven to be dimers on the basis of molecular weight and fragmentation obtained by ^{252}Cf-plasma desorption mass spectrometry (10) (Table 3). The amino acid analysis and elemental analysis (not listed) were also consistent (Table 3). The DPE$_n$ compounds were evaluated for their activity in three assay systems, employing ^{3}H-NAL, ^{125}I-DADLE (14), and ^{3}H-DAMEA as labeled ligands, to examine the μ receptors, the δ receptors, or a combination of μ and δ activity, respectively. The monomeric pentapeptide DALEA was used as a standard, since dimeric analogues DPE$_n$ can be regarded as two enkephalin amides linked by the methylene chain (Fig. 1).

Use of ^{125}I-DADLE as labeled ligand is thought to reflect δ receptor activity (14). In this assay system, the most potent analogue, DPE$_2$, appears to be approximately 7 times more active than the standard (DALEA), with a 7-fold reduction in the ED$_{50}$ for displacement of labeled ligand. As chain length increases to $n = 4$ and $n = 6$, there is only a slight decrease in activity, and even for $n = 10$ the dimeric enkephalin shows greater activity than the monomer. Compound DPE$_{12}$ shows a severe loss of activity, with activity only approximately 30% that of the monomeric DALEA. Figure 3A summarizes the relative activity as a function of chain length.

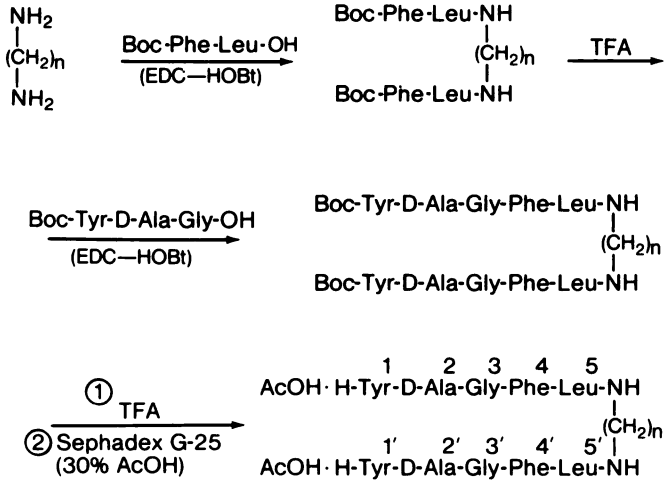

FIG. 2. *Synthesis of DPE$_n$*

When ^{3}H-NAL was used as labeled ligand, the dimeric enkephalins showed relatively unimpressive activity, and all DPE$_n$ compounds were less potent than the standard (Table 4, first column; Fig. 3A). For $n = 2$, 4, and 6, the DPE$_n$ showed activity between 80 and 100% that of DALEA, whereas DPE$_8$ was only 60% as potent. DPE$_{10}$ and DPE$_{12}$ showed significantly lower activity. The relationship of relative activity to the length of the cross-linking methylene chain (Fig. 3A) was rather flat.

When ^{3}H-DAMEA is utilized as labeled ligand, one obtains a combination of δ and of μ receptor binding activity (15). Table 4 summarizes the potencies of DPE$_n$ in such an assay. Again, DPE$_2$ was most potent, and DPE$_4$ and DPE$_6$ were also more potent than the standard DALEA. DPE$_8$ was approximately equipotent with DALEA, whereas DPE$_{10}$ and DPE$_{12}$ showed only 55% and 20% of the activity of the monomer. The activity-versus-chain length relationship in this assay system was nearly linear (Fig. 3A).

In order to compare the results from the three different binding assays, to examine further the effect of chain length, we normalized the data by dividing the ED$_{50}$ for any given compound by the corresponding ED$_{50}$ for DALEA in the same binding assay system (Fig. 3B). There was a progressive loss of activity with the increase in chain length for each of the three radioligand assays. All three assays show a similar pattern, although the absolute magnitude of change was most pronounced when ^{125}I-DADLE was used as labeled ligand, where activity changed 34-fold, and was minimal in the ^{3}H-NAL binding assay, where only a 5-fold change of potency was observed.

We sought to quantitate the selectivity of each of the new compounds (DPE$_n$), again as a function of chain length. A "selectivity ratio" was defined as the ratio of the ED$_{50}$ for any given compound, when ^{3}H-NAL was used as a tracer, relative to its ED$_{50}$ when ^{125}I-DADLE

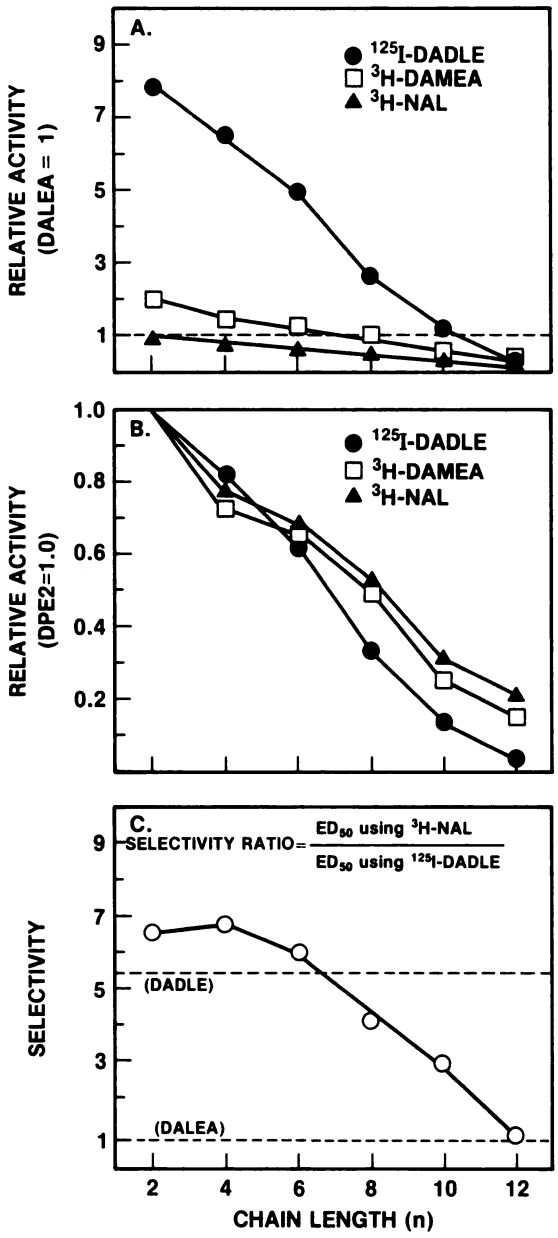

FIG. 3. *Relative activity of DPE$_n$ as a function of chain length*

A. Relative activity of DPE$_n$ in three radioligand assays as a function of chain length (n), expressed relative to DALEA.

B. Relative activity, expressed as percentage of activity for DPE$_2$, as a function of n.

C. Selectivity ratio, defined as ED$_{50}$ for any given compound in the ^{3}H-NAL assay, relative to its ED$_{50}$ in the ^{125}I-DADLE assay, as a function of chain length (n).

was used as labeled ligand (Fig. 3C). A completely non-selective compound with the same potency in both assays would have a selectivity ratio of 1 (16). The shorter the length of methylene chain, the higher the selectivity of the dimeric enkephalins for the δ receptor. Compounds DPE$_2$, DPE$_4$, and DPE$_6$ showed greater selectivity than the previously best-known δ specific ligand, DADLE. DPE$_8$ and DPE$_{10}$ showed progressively decreasing selectivity, whereas DPE$_{12}$ showed no selectivity for the δ relative to the μ receptor.

TABLE 4

Receptor binding activities of dimeric enkephalins (DPE$_n$)

Binding assays were carried out in rat brain membranes washed three times with Tris·HCl (50 mM, pH7.5) containing bacitracin (100 μg/ml), for 1 hr at 25°. All results were confirmed in duplicate experiments. Mean ED$_{50}$ was calculated assuming a log-normal distribution. The percentage error corresponds to ± 1 SEM for between-experiment variability, calculated assuming a log-normal distribution, and pooling estimates of the standard deviation of log ED$_{50}$ for all seven preparations tested.

Enkephalin	ED$_{50}$		
	^{3}H-NAL	^{3}H-DAMEA	^{125}I-DADLE
		nM	
Monomer			
DALEA	1.74	1.99	2.17
Dimers			
DPE$_2$	1.78	1.01	0.27
DPE$_4$	2.28	1.40	0.33
DPE$_6$	2.61	1.53	0.43
DPE$_8$	3.37	2.06	0.83
DPE$_{10}$	5.82	3.99	1.95
DPE$_{12}$	8.51	6.50	9.12
Relative % error	±22%	±28%	±37%

DISCUSSION

The synthesis of dimeric analogues by the conventional solution methods as described here offers several advantages. (a) Cross-linkages are built up by amide bond formation which makes the splitting of dimers by metabolic processes unlikely. (b) Synthesis of dimeric analogues can be carried out exactly like that of a monomeric analogue except that 2 equivalents of peptide acids are used. This means that a dimeric analogue can be obtained with essentially the same effort as monomeric pentapeptide enkephalins. We did not observe any difference of reactivity among the series of dimers. (c) Coupling reactions can be conducted by the water-soluble EDC-HOBt method and using the fragment intermediate Boc-Tyr-D-Ala-Gly-OH, which avoids the problem of racemization at the COOH terminus. This resulted in high yields of well characterized final products (Tables 1–3).

The new "family" of enkephalin analogues has been tested in three parallel binding assays with labeled ligands of different selectivity for opiate receptors. Selectivity ratio values obtained from binding assays are usually lower than those derived from *in vitro* bioassays (16); however, they reflect more closely differences in receptor affinities and are less subject to interference from proteolysis. Since data shown here were obtained from binding assays performed in the same tissue (rat brain) and under the same conditions of temperature, time, peptidase inhibitors, and receptor concentration for all the ligands used, it is unlikely that factors other than receptor affinity are responsible for the remarkable differences obtained. We have demonstrated that the monomers and dimers do not undergo significant degradation during the course of incubation at 25°.

Dimeric enkephalins show a characteristic profile of activity versus the chain length of the cross-linking meth-

ylene bridge, with apparently maximal activity for short chains. Furthermore, when compared with monomeric enkephalin, they appear to show highest activity in radioligand assays employing a ligand selective for the δ receptor (^{125}I-DADLE) and show least activity when assayed under conditions favorable to μ receptor activity (^{3}H-NAL).

It seems appropriate to consider possible mechanisms for the characteristic pattern of relative activity and selectivity versus chain length as shown in Fig. 3A–C. The hydrophobicity of the methylene bridge might increase nonspecific adsorption to membranes and thus enhance activity. However, we regard this as unlikely, since there is a dramatic loss of activity and selectivity as chain length (and hydrophobicity) increase. Furthermore, one may expect that nonspecific hydrophobicity effects would increase affinity under all conditions tested. Although the increase in potency is sharply pronounced when we use the δ-specific ^{125}I-DADLE, it is modest when we use a nonselective peptide (^{3}H-DAMEA) and there is a loss of potency when ^{3}H-NAL is utilized in the binding assay. Note that under the experimental conditions used here (25° in the absence of sodium ion) the binding of naloxone correlates extremely well with that of [^{3}H]morphine and other μ agonists (17).

Another hypothesis for the enhancement of the δ activity of the dimeric pentapeptide enkephalins might involve structural, conformational changes in the enkephalin due to the methylene bridge itself. However, all reported modifications of the carboxy group in the COOH-terminal residue (esters, amides), have consistently resulted in a loss of δ activity and increased activity for μ receptors (18–20). In contrast, in the present study, the linkage of two molecules of a relatively nonselective ligand (DALEA) (15) have resulted in very selective compounds. In our view, the most attractive explanation for the present findings is that the dimeric enkephalins might cross-link δ receptors, which are presumably clustered together with appropriate intermolecular distances, but fail to cross-link μ receptors, or do so with a much lower probability. The ability to bind simultaneously to two receptors would result in an increase in relative activity and a great increase in selectivity. If this hypothesis is correct, it would have significant implications for the organization of δ and μ receptors in the membrane. It is curious that the greatest activity and best selectivity are seen with the shortest methylene chain ($n = 2$). This would suggest that δ receptors might be extremely closely clustered in the membrane as indicated by Hazum *et al.* (21). Since the methylene bridges are flexible, an exact calculation of the distance between binding sites is not possible. Flexibility might also explain why a smooth decline in potency is obtained with increasing length rather than a sudden decrease after some threshold value.

We have also synthesized another dimeric enkephalin, 2,7-*N*,*N*′-bis ([D-Ala^2,Leu^5]enkephalin)-diamidofluorene, linked by a rigid fluorene moiety with a length approximately corresponding to $n = 6$ in the present series. This compound was essentially equipotent with DPE_{12} and showed no δ selectivity (data not shown).

A dimeric enkephalin has been synthesized previously (22) using solid-phase procedure and lysine in position 6 as connecting bridge. The resulting asymmetrical dimer has been reported more active than enkephalin standard *in vivo* but less potent in the vas deferens bioassay. However, its selectivity and/or binding activity have not been reported.

We have also initiated studies of some dimers of the metazocines, prepared by Drs. Herbert Merz and Everette L. May of the Medical College of Virginia. Three of the di-*N*-metazocinoalkanes did not display increased activity in the μ receptor assay. These compounds, and dimers of opiate alkaloids, are deserving of additional study.

The high δ activity of the new family of dimeric enkephalins presented here (especially DPE_2) suggests that these compounds should be very useful probes for studying δ receptors with the use of ligand binding and autoradiographic and *in vivo* techniques. Indeed, we have recently obtained considerable additional evidence confirming that the enkephalin dimers are active agonists for the δ receptor: (a) The ED_{50} for DPE_2 displacing ^{3}H-NAL is increased by a factor of 4 in the presence of 25 mM Na^+; (b) DPE_2 inhibits the prostaglandin E_1-stimulated increase in cyclic AMP in neuroblastoma glioma (NG108-15) cells,[2] with an IC_{50} of ~0.1 nM, similar to that of DADLE and considerably more potent than DALEA or DAMEA (~4 nM); and (c) DPE_2 shows increased potency (relative to DADLE) in the mouse vas deferens assay but not in the guinea pig ileum assay.

Further evidence consistent with the hypothesis that DPE_2 can attach to two δ receptors simultaneously is as follows: (a) The tritiated dimer ^{3}H-DPE_2 shows at least a 5-fold increase in affinity for the δ receptors of intact neuroblastoma glioma (NG108-15) cells, with a 2-fold increase in association rate and at least a 2-fold lower dissociation rate than DADLE or DAMEA; (b) use of newly synthesized dimers of the μ-selective *tetra*peptide H-Tyr-D-Ala-Gly-Phe-NH_2 results in a dramatic, 1000-fold shift in selectivity, with formation of a compound with high affinity ($K = 10^9$ L/M) for the δ receptor and 90-fold δ/μ selectivity when $n = 12$, such that the spacing between the two peptide moieties is comparable to that of DPE_2 (23).

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