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STRUCTURE-ACTIVITY RELATIONSHIPS OF ENKEPHALIN-LIKE PEPTIDES

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INTRODUCTION

The isolation, identification, and characterization of two naturally occurring pentapeptides, Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and the relationship of these peptides to other opiate-like peptides (endorphins) have been fully covered in other reviews (e.g. 1–3). Because of the unique situation in which peptides (e.g. enkephalin) and nonpeptides (e.g. morphine) appear to interact at the same receptor site, there has been interest in structure-activity (S-A) relationships on an unprecedented scale. Progress in interrelating peptide and nonpeptide structures is not covered in this review; despite many conformational and other studies no firm conclusions have yet emerged (see 3, 4). Likewise, discussion of the increasing interest in S-A relationships of endorphins of higher molecular weights (see 5–7) is omitted. This review is concerned with the effect on biological activity of molecular changes in Met/Leu-enkephalin based on results in in vitro and in vivo tests. Early data have been reviewed by Morgan (8).

Let us first consider some general principles pertaining to all S-A studies. Biological activity, as measured in even the simplest of in vitro systems, is the culmination of a series of molecular processes in which the compound under evaluation interacts with other molecules. Important interactions in the cases of test systems used in studies of enkephalin-like peptides are shown in Figure 1. The analysis of in vivo models applies to any test where centrally mediated effects are being measured, e.g. the mouse hot plate or

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rat tail flick test. A complex series of interactions determine the life of intravenously administered compounds and their ability to arrive at receptors in the central system (Figure 1, first column). When, in these in vivo tests a compound is administered intraventricularly (Figure 1, second column), the events in blood and the problems governing entry into the CNS are circumvented; however, transport in the CNS may be determined by factors different from those pertaining when entry is gained via blood, and metabolism in the CNS may still be important. Responses in common in vitro tests (guinea pig ileum, mouse vas deferens) (Figure 1, third column) may be initiated by interactions at more than one type of receptor, and metabolism of test compounds may be determined by enzymes specific to the test concerned. The situation in receptor binding studies (Figure 1, fourth column) is especially complex when peptides are being studied; all test systems contain proteases which may lead to extensive or partial metab-

	In vivo model		In vitro model	Receptor binding studies	Stability to	
	iv	icv			Blood	Brain extract
1 In blood compartment						
a) Loss, via excretion or uptake by tissues	✓	x	x	x	x	x
b) Re-uptake						
c) Metabolism in blood	✓	x	x	x	✓	x
d) Binding to plasma proteins	✓	x	x	x	x	x
2 Entry into CNS						
a) Blood-brain barrier						
b) Blood-CSF barrier	✓	x	x	x	x	x
c) Other means						
3 Transport in CNS after leaving blood	✓	(✓)	x	x	x	x
4 Metabolism by tissue enzymes	✓	✓	(✓)	(✓)	x	(✓)
5 Activity at receptor						
a) Affinity	✓	✓	(✓)	✓	x	x
b) Efficacy	✓	✓	(✓)	x	x	x
c) Stimulus-response coupling	✓	✓	(✓)	x	x	x

Figure 1 Molecular processes involved in the testing of analogues that act centrally in the CNS. In the first column (intravenous administration of analogues in an in vivo test), all processes are involved, as indicated by ticks; in other columns, processes may or may not be involved, as indicated by ticks or crosses. Ticks in parentheses indicate doubts as to relevance to a similar process listed in the first column.

olism, none of the methods used to reduce proteolysis is entirely satisfactory, and "high affinity" binding may not necessarily be associated with biological response. Each interaction in each test system may have distinct structural/conformational requirements leading to a series of structure-function relationships. The summation of these leads to S-A relationships in a given test under defined conditions. Conclusions about individual molecular processes from results in individual tests are clearly not justified, yet they have frequently been drawn. More meaningful conclusions arise when the results from biological tests are compared with those from supplementary tests designed to study specific processes. Only tests to follow metabolism in blood or tissue extracts (Figure 1, last two columns) have so far been employed. Assessment of the stability of peptides in blood is clearly of direct relevance, but the results with tissue extracts may inaccurately reflect stability toward tissue proteases of importance in biological tests (they probably more often reflect stability toward proteases that are released and are stable in the process of preparing the extract). It is to be hoped that such supplementary tests will be refined and extended in the future.

The number of enkephalin analogues described in the literature is now formidable, and most have been tested under a wide range of procedures and conditions. Data are most extensive from isolated tissue and opiate binding tests; in these cases a complete coverage of results with analogues derived (formally) by single change at each amino acid position of Met/Leu-enkephalin, and their simple ester or amide derivatives, has been attempted. In all other cases no attempt has been made to cover analogues exhaustively. Results are either treated generally, or selection has served to illustrate significant trends. Information disclosed in patent literature has usually been excluded.

ISOLATED TISSUE PREPARATIONS AND OPIATE RECEPTOR BINDING

The effect of structural change at each amino acid position has been studied using either Met-enkephalin, Leu-enkephalin, or a more stable analogue as the reference compound. For the same structural change, results with different reference compounds are not always similar, especially when comparing the cases of Met/Leu-enkephalin with stable analogues. Discussion is centered on the effect of *single* substitution (any one amino acid position varied, but not two) on biological activity in the two most commonly used preparations, i.e. the electrically stimulated mouse vas deferens (MVD) or guinea pig ileum (GPI) assays, using Leu/Met-enkephalin, or their simple ester or amide derivatives, as reference compounds. Each position (N-terminus, Tyr^1, Gly^2, Gly^3, Phe^4, Met/Leu^5) of Met/Leu-enkephalin is considered in

turn. The analogues on which discussion is based are listed in Tables 1–6, which should be read in conjunction with the text, and which contain the literature references to individual compounds. Distinction between results in the two assays is only made when they are at variance, or when only one set of results has been reported. In all these tables potencies of analogues have been related to Met-enkephalin (=1). Where analogues of Leu-enkephalin, or of Met- or Leu-enkephalin methyl ester or amide, are listed the potency of the reference compounds concerned can be found in Table 6. Values quoted have been calculated or estimated when Met-enkephalin has not been used as reference compound in the original paper. Data from opiate receptor binding studies are included (without any attempt to define conditions) for completeness, but not discussed. The effect of peptide backbone modification and multiple substitution are then considered briefly, and general conclusions are presented.

N-Terminal Substitution (Table 1)

The general conclusion (24) that "increasing the size of the pentapeptide by addition of amino acids to the N-terminal results in loss of activity" is incorrect. In the GPI assay, hexapeptide esters derived by addition of Lys, Arg, Gly, or Dab to the N-terminus of Leu-enkephalin methyl ester are respectively 1.5, 1.3, 1.1, or 1 times as active as Leu-enkephalin methyl ester (0.2 × Met-enkephalin), and analogous esters involving N-terminal Phe, D-Asp, or Pyr have significant activity. Likewise, addition of Arg, Gly, Phe, or Tyr to the N-terminus of Met-enkephalin causes very little loss of activity. Met-enkephalins extended at the N-terminus by more than one amino residue are generally weakly active; however, similar extensions of "stable" enkephalins may provide highly active analogues (25). In contrast, addition of acetyl, Boc, D-amino acids, or residues of the type NH_2-$(CH_2)_x$-CO(x = 2, 3, or 5) to the N-terminus of either Leu- or Met-enkephalin leads to inactive or very weakly active compounds, suggesting that exo-peptidases play an important role in determining the potency of N-terminally extended peptides. This seems a more likely explanation of the results (see Conclusions) than the alternative that has been advanced (13).

In the GPI assay, methylation of the terminal (Tyr) amino group of Met-enkephalin has little effect on potency, but similar methylation of Leu-enkephalin increases potency by a factor of 3.8. The effect of methylation is even more marked in the case of Leu-enkephalin methyl ester (analogue 13 times more potent than the parent compound). Dimethylation of the terminal amino group of Leu-enkephalin methyl ester causes a marked drop in potency. In the MVD assay, all the mono methylated analogues are less potent than the parent compound (by a factor of 2–5). Dimethylation causes an even more marked drop in potency.

Table 1 N-Terminally substituted derivatives. In vitro activity[a] of analogues of type X-Tyr-Gly-Gly-Phe-Y, where *Y* = Met or Leu

Analogue[b]		Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
X =	*Y* =				
Arg-	Leu-OH		0.078	0.04	9
	Leu-OMe	0.29			10
	Met-OH	0.90			11
	Met-OH	0.25			12
	Met-OH	0.19	0.22	0.075	13
	Met-OH			0.4	14
D-Asp	Leu-OMe	0.008			10
Dab-	Leu-OMe	0.20			10
Dab(Boc)-	Leu-OMe	0.084			10
Gly-	Leu-OMe	0.22			10
	Met-OH	0.19	0.30	0.15	13
Lys-	Leu-OMe	0.30			10
	Met-OH	0.20	0.25	0.075	13
Lys(Boc)-	Leu-OH	0.14			10
	Leu-OMe	0.34			10
D-Lys(Boc)-	Leu-OMe	0.004			10
Phe-	Leu-OMe	0.01			10
	Met-OH	0.21	0.25	0.1	13
Pyr-	Leu-OH	0.001			10
Sar-	Met-OH			0.01	14
Tyr-	Leu-OH			1.0	15
	Met-OH	0.15	0.28	0.1	14
Lys-Arg-	Met-OH	0.07			11
	Met-OH			0.2	13
Asp-Lys-Arg-	Met-OH	0.044			11
Lys(Glu-Gly)-	Leu-OMe	0.17			10
Lys-Asp-Lys-Arg-	Met-OH	0.003			11
Pro-Lys-Asp-Lys-Arg-	Met-OH	0.0011			11
MeCO-(Ac)	Leu-OH	<0.001			10
	Met-OH	"low"			16
Me_3C-O-CO-(Boc)	Leu-OH	0.002			10
	Leu-OH		0.0003		9
	Met-OH	0.002			10
NH_2-C(:NH)-NH-CH_2-CO-	Leu-OMe	0.07			10
NH_2-CO-(carbamoyl)	Met-OH			<0.0009	17
$PhCH_2$-O-CO-(Z)	Leu-OH		0.02		18
NH_2-$(CH_2)_2$-CO-(β-Ala)	Met-OH	<0.013	<0.0027	0.011	13
NH_2-$(CH_2)_3$-CO-	Met-OH	<0.013	<0.0027	0.0033	13
NH_2-$(CH_2)_5$-CO-	Met-OH	<0.013	<0.0027	0.0021	13
p-OH-C_6H_4-$(CH_2)_2$-CO-	Met-OH	<0.013	<0.0027	0.0019	13
CH_2: CH-CH_2-(allyl)	Met-OH	0.105			19
	Leu-OH	0.43			19
Methyl	Leu-OH	0.76			10
	Leu-OH		0.75	0.029	9

Table 1 *(Continued)*

Analogue[b]		Guinea pig	Mouse vas	Receptor	
X =	Y =	ileum	deferens	binding[c]	Reference
Methyl *(continued)*	Leu-OH	2.58			20
	Leu-OMe	2.6			10
	Leu-OMe		0.209	0.04	9
	Leu-OMe	2.08			20
	Leu-NH_2	1.3			10
	Leu-NH_2	4.85	0.85		21
	Met-OH	1.0	0.2		22
	Met-OH		0.21		23
	Met-OH	1.90			11
	Met-OH			4.5	17
	Met-NH_2	5.25	0.1		22
	Met-NH_2			3.0	17
Dimethyl	Leu-OH		0.0125	0.0057	9
	Leu-OMe		0.0219	0.0023	9
	Leu-OMe	0.08			10
Cyclopropyl, methyl	Met-OH	0.015	0.008		22

[a] Potencies in the guinea pig ileum, mouse vas deferens, and receptor binding assays relative to Met-enkephalin = 1.

[b] Abbreviations for amino acids and their use in the formulation of derivatives follow the various recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Boc = t-butoxycarbonyl.

[c] Comparison of data obtained by different investigators is seldom justified, since the source and preparation of tissue fractions or homogenates, the conditions of incubation in the assay, and the nature of the radiolabeled ligand vary widely.

Tyr[1] Position (Table 2)

The structural requirements at this position are clearly very precise in both assays. Thus, removal of the *p*-OH group (Phe analogue) in Met-enkephalin, of the amino group (des-NH_2-Tyr analogue) in Met- or Leu-enkephalin, or of the complete Tyr residue (analogue where $X = H$ in Table 2) leads to inactive analogues. Replacement of the *p*-OH group by a *p*-amino, -nitro, -chloro, or -iodo group [Phe(*p*-NH_2) etc], or of the amino group by aminoxy [OTyr analogue] likewise results in loss of agonist activity. Configurational requirements are also precise, as evidenced by the inactivity of the D-Tyr and α-Aztyr (α-CH replaced by N) analogues. Replacement of Tyr by other amino acids (Gly, Ala, D-Ala, His, Trp, Sar) likewise leads to inactive analogues.

Apart from the preserved or increased activity resulting from N-methylation (see preceding section) the only modifications that have provided analogues with significant activity are (*a*) contraction of the side chain of Tyr by omission of the methylene group [Gly(*p*-OH-phenyl) analogue], (*b*) extension of the peptide chain by means of a further methylene group (β-homo-Tyr analogue), (*c*) methylation of the *p*-OH group [Tyr(Me) analogue].

Table 2 Variation of tyrosine[1] residue. In vitro activity[a] of analogues of type X-Gly-Gly-Phe-Y, where Y = Met or Leu

Analogue[b, d]		Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
X =	Y =				
H-	Leu-OMe	<0.0002			10
	Met-OH	inactive			11
	Met-OH		0.001		26
Ala-	Met-OH	inactive			11
D-Ala-	Met-OH	inactive			11
Aztyr-	Leu-OMe	<0.0002			10
Gly-	Met-OH	inactive			11
Gly(p-OH-phenyl)-	Leu-OMe	0.002			10
His-	Leu-OMe	<0.0002			10
	Met-OH			<0.0004	27
Phe-	Met-OH	0.002	0.0003	0.001	28
	Met-OH	0.002	<0.0015		16
	Met-OH	<0.0015	<0.0015		29
	Met-OH			0.002	14
Phe(p-NH_2)-	Met-OH	<0.01			11
Phe(p-Cl)-	Met-OH	<0.01			11
Phe(p-I)-	Met-OH	<0.01			11
Phe(p-NO_2)-	Met-OH	<0.01			11
Phe(3,4-di-OH)-	Met-OH			0.004	14
Sar-	Met-OH			0.005	27
Trp-	Met-OH			0.0004	27
	Met-OH			0.0008	14
D-Tyr-	Met-OH	<0.01			11
	Met-OH		0.005		9
	Met-OH		<0.001	<0.01	30
Tyr(Me)-	Leu-OH		0.023	0.0046	9
	Met-OH	0.17			16
	Met-OH		0.004		23
Tyr(Bzl)-	Met-OH			very low	31
Tyr($CONH_2$)-	Met-OH			very low	31
OTyr-(aminoxy analogue)	Leu-OH		<0.001		—[e]
β-Homo-Tyr-	Leu-OH		0.037	<0.0023	9
des-NH_2-Tyr-	Leu-OMe	<0.0002			10
	Met-OH	inactive	inactive		28
	Met-OH	<0.0015			29

[a–c] See footnotes to Table 1.

[d] Aztyr = NH_2-N(CH_2-C_6H_4-OH)-CO-; OTyr = L-NH_2O-CH(CH_2-C_6H_4-OH)-CO-; β-Homo-Tyr = L-NH_2-CH(CH_2-C_6H_4-OH)-CH_2-CO-; des-NH_2-Tyr = HO-C_6H_4-$(CH_2)_2$-CO-; Sar = NHMe-CH_2-CO-.

[e] Unpublished.

Gly^2 Position (Table 3)

The introduction of D-amino acid residues in place of Gly^2 may cause a marked increase in potency in both assays. With Leu-enkephalin methyl ester as reference compound (0.2 X Met-enkephalin in GPI assay), the effect is optimal when Gly^2 is changed to D-Ser (55 times increase in potency), but residues approximately the size of D-Ser (D-Ala, D-Thr, D-Met) or of configuration intermediate between that of L- and D-residues (Azala) lead to analogues which are 15–40 times more potent than the parent compound. The D-Lys(Boc)^2, D-Phe^2, D-Leu^2, D-Asp^2, and D-Ser(Bu^t)^2 analogues are also all more potent than the parent compound, whereas the D-Lys^2 and D-Trp^2 analogues are less potent. The effect of D-substitution is less pronounced with Met-enkephalin as the reference compound. Only the D-Ala^2 analogue is reported to be more potent than Met-enkephalin (different authors report 1.14–6.25 times more in the GPI, and 5–10 times more in the MVD assays). In this case, the D-Ser^2 analogue is rather less potent than the parent compound. Interestingly, D-Ser^2 substitution in Met-enkephalin methyl ester causes a marked increase in potency (MVD assay; comparable figures from GPI assay not available), and the same effect is seen with Met-enkephalin amide as reference compound.

In contrast, the introduction of L-amino acid residues (Ala), or of L- or D-Pro, in place of Gly^2 causes a marked fall in potency in the cases of all reference compounds. Replacement of the CH_2 of Gly^2 by NH (Azgly^2 analogue), internal extension of the residue by CH_2 (β-Ala analogue), or methylation of the NH (Sar analogue) likewise causes a marked fall in potency, but disubstitution on the CH_2 by methyl (Aib analogue) results in only a small fall.

Gly^3 Position (Table 4)

With one exception, structural or conformational change at the Gly^3 residue leads to a drop in potency, which in most cases is marked or complete. The exception is the change of CH_2 by NH, which provides an analogue (Azgly^3) which is four times more potent than the parent in the GPI assay. Analogues of both Met- and Leu-enkephalin with Gly^3 substituted by Ala or D-Ala are only weakly active. Methylation of the NH gives inactive compounds (Sar analogues). Other changes (followed with Leu-enkephalin or its methyl ester) providing very weakly active, or inactive, analogues are L- or D-Pro replacement, elimination of the residue (des-Gly analogue), and extension of the residue by inserting an additional CH_2 (β-Ala analogue) or by intercalating Gly (Gly-Gly analogue).

Phe^4 Position (Table 5)

The Gly^4, Ala^4, D-Ala^4, D-Phe^4, Tyr^4, and D-Trp^4 analogues of Met-enkephalin are virtually inactive, but the Trp^4 analogue has appreciable activity.

Table 3 Variation of glycine[2] residue. In vitro activity[a] of analogues of type Tyr-X-Gly-Phe-Y, where *Y* = Met or Leu

Analogue[b, d]					
X =	*Y* =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
Aib-	Leu-OH	0.13			10
Ala-	Leu-OH		0.0094	0.004	9
	Leu-OMe	0.026			10
	Met-OH	0.064			11
	Met-OH			0.009	17
	Met-OH			0.05	32
	Met-NH_2			0.05	27
D-Ala	Leu-OH	1.83	8.3	6.25	9
	Leu-OH	2.30			11
	Leu-OH	3.05	9.4		33
	Leu-OH	2.65			25
	Leu-OMe	5.6			10
	Leu-OMe		2.97	2.22	9
	Leu-NH_2	5.0	1.6		22
	Leu-NH_2	4.8			25
	Met-OH	6.25	5.0		22
	Met-OH	1.14	7.2	5.26	9
	Met-OH	3.16			11
	Met-OH		10.0	1.20	30
	Met-OH		5.0		26
	Met-OH			0.54	32
	Met-OH			0.5	27
	Met-OMe	4.2			25
	Met-OMe		2.94	4.0	9
	Met-NH_2	8.0	1.9		22
	Met-NH_2	7.75	3.81		21
	Met-NH_2		1.0	1.50	30
	Met-NH_2			0.5	27
β-Ala-	Leu-OH	0.002			10
D-Asp-	Leu-OMe	0.76			10
Azgly-	Leu-OMe	0.02			10
Azala	Leu-OMe	5.7			25
D-Leu	Leu-OH		0.20		9
	Leu-OMe	1.34			10
	Met-OH			0.01	30
D-Lys	Leu-OMe	0.18			15
D-Lys(Boc)-	Leu-OMe	1.9			10
D-Met-	Leu-OMe	63.0	2.99		21
	Leu-OMe	8.0			10
	Leu-NH_2	27.5	3.68		21
	Leu-NH_2	9.0			25
D-Phe-	Leu-OMe	1.32			10
	Leu-OH	0.54			—[e]
	Met-OH			0.01	30
	Met-NH_2			0.1	27

Table 3 *(Continued)*

Analogue[b, d] X =	Y =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
Pro-	Leu-OH		0.0003	0.0023	9
	Met-OH			0.009	8
	Met-NH_2			0.0022	27
D-Pro-	Leu-OMe	0.002			10
	Met-OH	0.01			11
Sar-	Leu-OH		0.0037	0.0023	9
	Leu-OH	0.014			—[e]
	Leu-OMe	0.014			10
	Met-OH	0.021			11
	Met-OH		0.006		34
	Met-OH			0.3	8
	Met-NH_2			0.025	27
D-Ser-	Leu-OMe	11.0			10
	Met-OH	0.91			11
	Met-OMe	5.16			—[e]
	Met-OMe	14.3	29.5		21
D-Ser(Bu^t)-	Leu-OH	0.64			10
D-Trp-	Leu-OH		0.05	2.5	9
	Leu-OMe	0.06			10
D-Thr-	Leu-OMe	3.0			10
	Met-OH	0.56			11
D-Val-	Met-OH	0.422			11
	Met-NH_2			0.05	27

[a-c] See footnotes to Table 1.

[d] Aib = -NH-CMe_2-CO-; Azgly = -NH-NH-CO-; Azala = -NH-NMe-CO-; Sar = -NMe-CH_2-CO-.

[e] Unpublished.

The Azphe[4] (α-CH of Phe replaced by N) or MePhe[4] (NH of Phe methylated) analogues of Leu-enkephalin are also highly active (potency 0.25 that of Leu-enkephalin in GPI assay). It is, however, incorrect to assume from these results that an aromatic ring is necessary for the display of activity; while the hexahydro-Phe (aromatic ring fully saturated) derivatives of Leu- or Met-enkephalin have not been described, the hexahydro-Phe derivatives of [D-Ala][2]-Leu-enkephalin methyl ester and [D-Met[2], Pro[5]]-enkephalin amide are appreciably active (25, 36). Apparently *p*-substitution of the aromatic ring also affords active analogues. The results are documented only in the D-Ala[2] series, the order of potency being -NO_2, -Cl,-Br (very effective) > -CF_3,-SO_2Me, -SOMe, -SMe (moderately effective) > NH_2, OH (inactive) (R. J. Miller, personal communication; see also 37, 38). One change, also documented only in the D-Ala[2] series, results in increased potency; [D-Ala[2], Δ^zPhe[4]]-Met-enkephalin amide (wherein

Table 4 Variation of glycine[3] residue. In vitro activity[a] of analogues of type Tyr-Gly-X-Phe-Y, where Y = Met or Leu

Analogue[b, d]		Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
X =	Y =				
Ala-	Leu-OH		0.025	0.0023	9
	Met-OH	<0.01			11
D-Ala-	Leu-OH	0.018			10
	Leu-OH		0.0031	0.0004	9
	Met-OH	0.04			16
	Met-OH		<0.001	0.1	30
	Met-OH		0.0016	0.0023	9
β-Ala-	Leu-OH	<0.001			10
Azala-	Leu-OMe	0.06			10
Azgly-	Leu-OMe	0.8			10
des-Gly	Leu-OH	0.0018			35
	Leu-OMe	<0.001			10
Pro-	Leu-OH		0.00031	0.00057	9
D-Pro-	Leu-OH		0.0053	<0.0023	9
	Leu-OMe	<0.001			10
Sar	Leu-OH		0.0031	0.022	9
	Leu-OMe	<0.001			10
	Met-OH	<0.001			34
Gly-Gly	Leu-OH	0.00625			35

[a-c]See footnotes to Table 1.
[d]Azgly = -NH-NH-CO-; Azala = -NH-NMe-CO-; Sar = -NMe-CH_2-CO-.

unsaturation has been introduced at the α-carbon atom) is five times more potent than [D-Ala[2]]-Met-enkephalin amide in the GPI assay (39).

Met/Leu[5] Position (Table 6)

While only modest increases in potency have been reported as a result of structural/conformational change at the Met/Leu position, analogues are invariably active. Indeed the tetrapeptide, Tyr-Gly-Gly-Phe (des-Leu/Met analogue), in which the Met/Leu residue has been removed, has significant (albeit weak) activity in both assays. Further contraction to the des-carboxy tetrapeptide also provides active analogues and is associated with interesting selectivity toward the GPI assay; Tyr-Gly-Gly-NH$(CH_2)_2$Ph has not been described, but Tyr-D-Ala-Gly-NH$(CH_2)_2$Ph is almost as potent as [D-Ala[2]]-Met-enkephalin in the GPI assay, and only very weakly active in the MVD assay (22).

In the GPI assay, the most potent analogue is probably the Met-NH_2[5]; alkylation of the amide (Met-NHEt analogue) reduces the potency slightly, and the D-Met-NH_2 analogue is appreciably less potent (equal to that of the D-Met analogue, i.e. 0.1 times Met-enkephalin). The potency of similar

Table 5 Variation of phenylalanine[4] residue. In vitro activity[a] of analogues of type Tyr-Gly-Gly-X-Y, where Y = Met or Leu

Analogue[b, d]		Guinea pig	Mouse vas	Receptor	
X =	Y =	ileum	deferens	binding[c]	Reference
Ala-	Met-OH	inactive			11
D-Ala-	Met-OH	<0.01			11
Azphe-	Leu-OH	0.05			10
Gly	Met-OH	inactive			11
	Met-OH	<0.0001			34
MePhe-	Leu-OH	0.05			10
D-Phe-	Met-OH	<0.01			11
	Met-OH		0.0006		34
	Met-OH		<0.001	<0.01	30
	Met-OH		0.00024	<0.0012	9
	Met-OH			<0.01	32
Tyr	Leu-OH		0.016	0.0011	9
	Met-OH		0.001		28
Trp	Leu-OH	low activity			35
	Leu-OH			0.32	32
	Met-OH		0.27		23
	Met-OH			1.29	32
D-Trp	Met-OH			<0.001	32

[a–c]See footnotes to Table 1.
[d]Azphe = NH-N($CH_2C_6H_5$)-CO-; MePhe = -NMe-CH($CH_2C_6H_5$)-CO-.

analogues in the Leu series follows the same order, i.e. Leu-NH_2 > Leu-NHEt > Leu > D-Leu-NH_2 ≃ D-Leu, though the difference between Leu-enkephalin and the D-Leu/D-Leu-NH_2 analogues is much smaller than that between Met-enkephalin and its D-Met/D-Met-NH_2 analogues, and potency reaches only that of the Met series when, additionally, the α-amino group is methylated (MeLeu-NH_2 analogue). Reverting again to the Met series, sulfoxide formation [Met(O)-OH[5] analogue] reduces potency by a factor of 5, and sulfone formation [Met(O_2)-OH[5] analogue] causes a marked drop in potency; contraction of the side chain [Cys(Me) analogue] or redisposition of the S atom [Cys(Et) analogue] provides analogues which are also less potent. The potency of Leu-enkephalin or its methyl ester is about 0.2 times that of Met-enkephalin. Replacement of the α-CH by N (Azleu analogue) or methylation of the α-amino group (MeLeu analogue) causes a 2–3-fold increase in potency. In both series, removal of the C-terminal carboxy group [NH-$(CH_2)_2$-SMe and NH-$(CH_2)_2$-$CHMe_2$ analogues] provides weakly active analogues, but the des-carboxy Azleu analogue [-NH-NH-CH_2-$CHMe_2$] is as potent as Leu-enkephalin. When the Met/Leu residues are substituted by other amino acid residues potency follows the order Met > Nle > Leu > D-Leu > D-Met > D-Ala > L-Ala > Gly.

Table 6 Variation of methionine/leucine[5] residue. In vitro activity[a] of analogues of type Tyr-Gly-Gly-Phe-X

Analogue[b, d] X =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
Leu-OH	0.2	0.5		40
		1.6		33
	0.2			10
	0.37	1.7		28
	0.36	1.19	1.82	9
	0.17			12
	0.38			32
Leu-OMe	0.2			10
		0.278	0.33	9
Leu-NH_2	0.45			11
	0.56			10
			0.10	14
Leu-NHEt	0.34			10
Leu-ol		0.125		9
D-Leu-OH	0.15			11
		1.4	0.8	9
D-Leu-NH_2	0.13			11
Met-OMe		0.156		9
Met-NH_2	1.30	0.53		29
	3.02			16
		0.08		9
			3.0	8
			2.0	14
Met-NHMe		0.24		29
Met-NHEt	2.46			16
Met-ol			8.2	41
Met(O)-OH	0.20			16
		0.67		23
		0.35		9
Met(O_2)-OH	0.01			11
D-Met-OH	0.105			11
		0.002	0.1	30
D-Met-NH_2	0.105			11
Ala-OH	0.029			10
		0.0031	0.0034	9
D-Ala-OH	0.065			10
Azleu-NH_2	0.56			10
Cys(Me)-OH	"lower than			42
Cys(Et)-OH	Met-enk"			42
Gly-OH	<0.01			11
	0.02	0.03		28
		0.0031	0.0023	9
Ile-OH		0.78		9
MeLeu-OH	0.74			11
	0.3			10

Table 6 *(Continued)*

Analogue[b, d] X =	Guinea pig ileum	Mouse vas deferens	Receptor binding[c]	Reference
MeLeu-NH_2	2.74			11
Nle-OH	0.45			12
	0.5			43
	0.3			10
		0.16	0.15	9
Nle-OMe	0.36			10
	0.9			43
Phe-OH		0.11		34
Pro-OH	0.005	0.001		—[e]
Pro-NH_2	0.02			10
Pro-NHEt	0.86			10
Thr-OH			0.02	14
Val-OH		0.041	0.15	9
Met-Thr-OH		0.69		9
			0.84	32
			0.20	14
Leu-Tyr-Gly-OH		0.28	0.02	9
Met-Thr-Ser		0.52		18
Met-Thr-Ser-Glu-Lys			1.0	15
OH(des-Met/Leu)	0.004			10
	<0.01			11
		0.00625		9
			0.07	15
			0.02	32
NH_2(des-Met/Leu, amide)		0.005		29
NH-$(CH_2)_2$-SMe(des-CO_2H-Met)		0.07		29
NH-$(CH_2)_2$-$CHMe_2$(des-CO_2H-Leu)	0.006			10
NH-$(CH_2)_4$-Me(des-CO_2H-Nle)	0.2			10
NH-NH-CH_2-$CHMe_2$	0.2			10

[a-c] See *footnotes to* Table 1.

[d] Leu-ol = L-NH-CH(CH_2-$CHMe_2$)-CH_2OH; Met-ol = L-NH-CH(CH_2-CH_2-SMe)-CH_2OH; Met(O) = Met S-oxide; Met(O_2) = Met sulfone; Azleu = NH-N(CH_2-$CHMe_2$)-CO-; MeLeu = L-NMe-CH(CH_2-$CHMe_2$)-CO-.

[e] Unpublished data supplied by S. Bajusz.

In the MVD assay, Leu-enkephalin is usually reported to be rather more active than Met-enkephalin. The D-Leu[5] analogue is equally potent, but the D-Met[5] analogue is only very weakly active. All other analogues, including the amides, are less potent. The approximate order of potency is Leu ≃ D-Leu > Met > Ile > Met-NH_2, Met(O) > Leu-OMe > Met-NHMe > Nle > Met-OMe > leucinol > Phe > Leu-NH_2 > des-carboxy-Met > Val > Gly, Ala > D-Met. Of three C terminally extended analogues, the Met-Thr has highest potency (0.69 times that of Met-enkephalin).

Modification of the Peptide Backbone

The effect of interpolation of methylene at the Gly^2 and Gly^3 positions (β-Ala^2 and β-Ala^3 analogues), of N-methylation of the amide bonds, and of α-aza substitution ($Azgly^2$, $Azgly^3$, $Azphe^4$, $Azleu^5$ analogues) has been discussed above under appropriate headings. "Depsipeptide" analogues (CO-NH replaced by CO-O) have not yet been described. The effect of replacement of CO-NH by CH_2-CH_2 was explored at the Gly-Gly bond [$NH(CH_2)_4$-CO, i.e. aminovaleryl, in place of Gly-Gly] (10); the resulting analogue was weakly active in the GPI assay. Similar replacement at the Tyr-Gly bond, and replacement of NH or CO only by CH_2 (CO-NH replaced by CO-CH_2 or CH_2-NH) at various positions have also been described (44); two analogues of the latter type involving the Tyr-Gly bond (and Met-NH_2 or Met-ol at the 5 position) have high activity in the GPI assay (44). Fully retro Met-enkephalin (Met-Phe-Gly-Gly-Tyr) and the retro-enantiomer (D-Met-D-Phe-Gly-Gly-D-Tyr) are inactive (45); in these analogues the direction of the amide bonds are reversed (CO-NH replaced by NH-CO) as compared with Met-enkephalin, but the end groups (NH_2 and CO_2H) are also interposed. Results with retro analogues wherein the end groups are simulated, or with partial retro-analogues (46), are awaited with interest.

Multiple Changes

The term *multiple change* is applied when, starting from Leu/Met-enkephalin, more than one change in structure is made at a time. Most analogues showing activity in analgesic tests are so derived. There is a large number of analogues in this category, the commonest being of the type Tyr-X-Gly-Phe(or MePhe)-Y, where *X* equals D-Ala or D-Met, and *Y* equals a wide range of amino acid residues and their derivatives, or other organic radicles. However, in these cases the effect of structural change has seldom been followed systematically.

The most comprehensive set of published data deals with results in the GPI assay (25). Tyr-D-Ala-Gly-Phe-Leu-OMe (potency 5.6) (relative to Met-enkephalin = 1) and Tyr-D-Ala-Gly-Phe-Pro-NH_2 (potency 11.8) were chosen as reference compounds, and the effect of changes in structure, made one at a time, was studied. Most of the general conclusions deduced from work with Met-Leu-enkephalin as reference compound were confirmed (e.g. importance of Tyr^1 and Gly^3 residues), but many substitutions were found to have opposite effects in different reference compounds. For example, substitution of Leu in place of Met in Met-enkephalin or its methyl ester results in a fivefold drop in potency, whereas similar substitution in [D-Ala^2]-Met-enkephalin methyl ester results in an increase in potency. Likewise, D-Ser in place of D-Ala can cause opposite effects; Tyr-D-Ser-

Gly-Phe-Leu-OMe (potency 11) is more potent than Tyr-D-Ala-Gly-Phe-Leu-OMe (potency 5.6), whereas Tyr-D-Ser-Gly-Phe-Pro-NH_2 (potency 0.8) is much less potent than Tyr-D-Ala-Gly-Phe-Pro-NH_2 (potency 11.8). These results represent conspicuous failures of the "additivity rule" (47).

The fullest set of results in the MVD assay also deals with variants of [D-Ala^2]-enkephalins (9). In this assay the effect of structural change in the D-Ala^2 series is quantitatively much closer to that found in the Gly^2 series. One interesting exception is the large increase in potency resulting from change of Leu^5 to D-Leu^5; in the Gly^2 series the change causes only a 1.18 times increase, whereas in the D-Ala^2 series the increase is almost fourfold.

Results in both assays with analogues having highest potency in analgesic tests are given in Table 7. Unlike the situation with classical opiate agonists, there is no correlation between the potencies of analogues in the GPI, MVD, or receptor binding assays and their analgesic activity. Attempts have been made to relate the ratio of their potencies in the ileum and vas assays to their analgesic potency (48, 49); analogues with weak analgesic potency generally resemble Leu/Met-enkephalin in being more potent in the vas than in the ileum assay, whereas analogues with high analgesic potency tend to be equipotent in both assays (through increased potency in the ileum). This has led to speculation that analgesia is mediated via μ receptors of postulated greater relative abundance in the ileum than in the vas (see Conclusions for comment).

Conclusions

There is agreement among investigators as to the main features of S-A relationships in both assays. Of the five amino acid residues, the structural and configurational requirements at Tyr^1, Gly^3, and Phe^4 (in this order) are most stringent. The only changes at Tyr^1 that provide more potent analogues are N-methylation and, in some cases, N-extension by amino acid residues. Most analogues are inactive. The requirements at Gly^3 and Phe^4 are also precise. A little more configurational freedom is permitted (Azgly^3 and Azphe^4 analogues active), but the substitution of D-amino acid residues is not. N-methylation of Phe^4 (but not of Gly^3) causes only a small drop in potency, the aromatic ring of Phe^4 is not sacrosanct, and the Trp^4 analogue has activity, but most substitutions lead to inactive or weakly active analogues. Much more latitude is allowed at the remaining two positions (Gly^2 and Met/Leu^5). The dominant feature at the Gly^2 position is the large increase in potency (both assays) resulting from substitution of certain D-amino acid residues (D-Ala, D-Met, D-Ser). The Met/Leu^5 position can be varied widely without associated elimination of activity; how-

ever, resulting analogues are usually less potent than the parent compound, the exceptions being amides (GPI assay) and D-Leu^5 analogues (MVD assay), which are often more potent.

Considerable problems arise in attempting to interpret the data at a molecular level. A crucial question concerns the nature of the activity being measured. "Activity," in some measure, undoubtedly reflects the ability of analogues to intereact with receptors concerned with the observed biological response. But to what extent is it also a measure of the ability of analogues to be degraded by peptidases (or other enzymes) present in the preparations, or their ability to be transported to these receptors (see Figure 1)? There is no doubt that both preparations do contain enzymes which are capable of degrading Leu/Met-enkephalin and many of the analogues. Attention has been mainly focused on those (arising from broken cells and plasma, and present in both the tissue and the surrounding organ bath) which degrade by cleavage of the N-terminal Tyr residue. These "released" amino peptidases may account for the majority of enzymic activity in the preparations, but they are probably not specific enkephalin degrading enzymes (50). The argument (50) that enkephalin degradation is not a source of errors in the MVD and GPI assays because (*a*) the depressant effects of agonists are achieved in less than one minute, and (*b*) the organ bath serves as an inexhaustible reservoir of exogenously added agonist, may be valid for degradation by "released" enzymes, but does it hold for degradation by membrane-bound enzymes? It seems likely [direct evidence (51–53), and as a necessary corollary of the postulated neuromodulatory role of enkephalins] that membrane-bound enzymes do exist in the vicinity of opioid receptors, and that their specific function is to inactivate the enkephalins. The effective concentration of agonist at the receptor will then be determined by the equilibrium between agonist and inactivated agonist, controlled by such enzymes, as well as by the concentration of agonist that arrives in the vicinity of the receptor (receptor compartment).

The S-A relations discussed above lend strong support to the conclusion that, in both assays, aminopeptidase, carboxypeptidase, and probably endopeptidase activity play a role in determining observed activity. It may be further speculated that aminopeptidase activity is of major importance in the GPI assay, that the endopeptidase activity is mainly cleavage of the Gly-Phe bond, and that the main controlling enzymes are membrane bound. This does not preclude the possibility that certain structural changes (e.g. D-amino acids for Gly^2 in both assays, and for Met/Leu^5 in the MVD assay) also favor involvement in a receptor interaction (e.g. affinity). However, in these circumstances only the following conclusions about the nature of the receptor interaction(s) seem justified. The "minimal fragment" (if such a term has meaning) of enkephalin for interaction may be defined as

the descarboxy tetrapeptide Tyr-Gly-Gly-NH$(CH_2)_2$Ph. The presence of N-terminal amino (or alkylamino) and tyrosine hydroxyl, their correct spacial disposition, and the correct spacial disposition of the Tyr and phenyl rings are essential. The peptide backbone serves to promote correct spacing, but the peptide bonds themselves are not involved. Receptor binding assays confirm these general conclusions but, in the opinion of the reviewer, provide no further information, since interpretation of results is again complicated by metabolic and other factors [for discussion of the data from receptor binding studies see references (3, 8)].

Stripped of all elaboratory comment, essentially the same conclusions have been reached about the nature of the receptors involved in the action of nonpeptide opiates in these assays. What then is the justification for current speculation that multiple receptors (μ, κ, σ, δ) exist? [They could arise by elaboration of the basic structural features (e.g. additional affinity sites).] In the MVD assay, K_e values of antagonists (e.g. naloxone) against opioid-like agonists do differ significantly, under circumstances in which classical considerations lead to the conclusion that different receptors must exist (21); in brain homogenates, inactivation of opiate receptors by phenoxybenzamine is prevented selectively by preincubation with opiates or opioid peptides (54). This seems convincing evidence for the existence of two receptors (μ and δ) in vasa deferentia and brains. The possible influences of membrane bound deactivating enzymes in the vicinity of the receptors should, however, not be ignored in reaching conclusions of this nature. The pharmacodynamics of the receptor interaction is then complicated by the existence of additional binding sites (on the enzyme) which, while not directly coupled to response (and which are therefore not receptor binding sites), nevertheless affect response indirectly via the receptor-enzyme complex.

OTHER IN VITRO EFFECTS

The inhibition of intracellular levels of cAMP (basal, or after PGE_1 stimulation) in neuroblastoma × glioma hybrid cells has been followed using a restricted range of analogues (55, 56). In general, the results relate well with those obtained in the MVD assay; the potency of Leu- and Met-enkephalin is high (only the Nle^5 analogue is more potent), and the potency of analogues relates to structure in a similar way in both assays. The most interesting difference is the failure of D-Ala^2 substitution to cause an increase in potency in the hybrid cell assay. As in the case of the MVD assay, morphine is much less potent than Met- or Leu-enkephalin, and this has led to speculation (55) that the receptor sites involved in the assay are "enkephalin sites" with high affinity for the peptides but low affinity for nonpeptide

opiates. However, unlike the situation in the MVD assay, the K_e values for naloxone against Met-enkephalin and morphine are similar in the hybrid cell assay.

IN VIVO EFFECTS

Enkephalin-like peptides have been shown to exhibit all the diverse effects of opiate drugs. In the case of Leu/Met-enkephalin these can be demonstrated only under special conditions, but analogues have evolved which are active in all standard tests by any route of administration. There are now analogues which are considerably more potent than morphine following intravenous or subcutaneous administration, and appreciably more potent following oral administration. However, in the evolution of analogues there is presently little published information relating to what may be termed a ground rule in S-A studies, i.e. the effect on activity in a given test of structural changes made one at a time. Analgesic tests have been studied most, so these are dealt with more fully. In other tests, the performance of analogues found most potent in analgesic tests has usually been examined, so few conclusions can be drawn. Throughout discussion, potencies of analogues are expressed on a molar basis relative to morphine (=1). Since this has sometimes involved calculation from data presented in original papers, advance apologies are presented to authors if small inaccuracies have arisen.

Analgesia

The mouse tail flick or hot plate tests have been commonly used as models. Potencies of the most active analogues (*1–7*), and three other analogues (*8–10*), in these two tests are given in Table 7. The most potent, following different routes of administration, are *2* (subcutaneous), *1, 3, 4,* and *6* (intravenous), and *2* and *5* (oral). It is instructive to see how they evolved. The apparent failure of in vitro results (from GPI, MVD, and opiate receptor binding studies) to predict in vivo analgesic potency after central administration was first attributed to rapid degradation of the peptides by brain enzymes (brain extracts were indeed found to contain enzymes that caused rapid degradation of Met/Leu-enkephalin). The finding of potency approaching that of morphine in enzyme-resistant analogues (e.g. analogue *8*) supported this conclusion. However, such analogues, derived by D-Ala^2 substitution, were not active at relatively high doses after intravenous administration, and this was attributed to failure of the peptides to cross the blood-brain barrier (65). Entry from blood into the central system is, of course, one important parameter to be considered, but it is now clear that many of the other parameters listed in the first column of Figure 1 are

of equal importance in determining potency by this route. There are also further factors (e.g. entry into blood, absorption from GI tract, diffusion from subcutaneous sites) to be considered in arriving at subcutaneously or orally active analogues. The groups of Pless and Roemer, Bajusz and Ronai, Morgan and Metcalf, and Li have been most successful in resolving these problems. Their major findings are as follows. Stability to enzymes in body fluids and tissues is, of course, an essential factor (but the relevant enzymes in tissues may be membrane-bound—so the results of in vitro experiments where analogues are incubated with tissue extracts must be treated cautiously). This consideration seems to have dominated the work of Roemer & Pless (57), who looked for structural changes that resulted in "the formation of longer acting and orally active (i.e. *more stable*) analogs" (reviewer's italics). Their conclusions were that "the substitution of Gly in position 2 by D-Ala, N-methylation of Tyr and Phe in position 1 and 4 respectively, and the conversion of Met in position 5 to the corresponding alcohol results in stable, highly potent analogs." However, in an elegant study by Bajusz et al (68) analogues of simular resistance to enzymic degradation by human serum, rat brain extracts, or aminopeptidase varied markedly in their analgesic potency. A particularly interesting example is the comparison of data for Met-enkephalin and Pro^5-enkephalin; the latter was found to be rather more vulnerable to enzymes (and less potent in in vitro assays), yet it was much more potent (ED_{50} 64 $\mu M/kg^{-1}$) after intravenous injection in the tail flick test (when Met-enkephalin is virtually inactive). Bajusz et al concluded that the significance of increased enzyme resistance is overstated in the literature and that there are three other requirements which must be satisfied for high potency, i.e. "favourable transport properties, ability to cross the blood-brain barrier, and enhanced or improved binding capacity." Contrary to previously expressed opinions, overall results in all studies with enkephalin analogues indicate that lipophilicity per se is not important in the penetration of the blood-brain barrier by peptides; more probably, active transport mechanisms exist, and it is surprising that these have not yet been studied more systematically. To Bajusz's list may be added the ability to permeate other "barriers," and favorable binding to plasma proteins (the significance of which may extend beyond "transport"). It may be speculated that the conclusions of Roemer & Pless in respect of the 1–4 positions of enkephalins accurately reflect the requirements for enzymic stability and the receptor interaction, i.e. that the 1–4 tetrapeptide is the unit of structure which is concerned with the receptor interaction, and that it must be stabilized to enzymic attack by structural change that does not impede this interaction. But modification of position 5 may be the additional key to optimization of other factors. From the results in Table 7, it would appear that 5-position modifications that have most successfully achieved this are

Table 7 Molar potencies of enkephalin analogues relative to Met-enkephalin (in vitro tests) or morphine (in vivo tests)[a]

Analogue[b]	In vitro results (Met-enk = 1)			In vivo results (morphine = 1)								
				Tail flick test				Hot plate test				
	GPI	MVD	Reference	icv	sc	iv	po	icv	sc	iv	po	Reference
1. D-Ala^2,MePhe^4,Met(O)-ol^5	21.2	0.94	(33)	10^3	3.2	6.4	0.32		3.1		0.45	(37, 41)
2. MeTyr^1,D-Ala^2,MePhe^4,Met(O)-ol^5					16.4	4.9	1.6					(57)
3. D-Met^2,Pro-NH_2^{5}	9.3	0.33	(58)	78	1.6	5.9		25				(59)
	29.2	0.97	(49)							1.7		(49)
4. MeTyr^1,D-Met^2,Pro-NH_2^{5}	45.2	2.2	(49)							2.2		(49)
5. D-Thr^2,Thz-NH_2^{5}				27	1.5	4.8	1.7					(60, 61)
6. Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$-N(O)Me_2	6.5	0.18	(62)			7						(62)
7. D-Ala^2,MeMet-NH_2^{5}		5.0	(63)					238	0.3[c]			(64)
		2.6	(64)					144	4.0[d]			(64)
8. D-Ala^2,Met-NH_2^{5}	8.0	1.9	(22)	~1		<0.03						(65)
	7.7	3.8	(49)	0.16		<0.08						(66)
										<0.03		(49)
9. D-Ala^2,D-Leu^5	3.3	27.2	(33)	0.15		<0.08						(66)
				0.32								(67)
						≐0.4						(60)
10. D-Ala^2,D-Leu-NH_2^{5}				3.8		0.30						(66)
Morphine	2.2	0.03	(33)									

[a] Reference numbers are listed in parentheses.
[b] Thz = L-thiazolidine-4-carboxylic acid.
[c] Licking used as end-point in determining reaction time.
[d] An attempt to jump off the hot plate used as end-point.

substitutions by Pro-NH_2, Met-ol, 2-amino-thiazolidine-5-carboxamide, and N,N-dimethyl-N-oxyammonioethylamino. Finally, attention is drawn to the remarkable potency (several thousand times that of morphine) of analogue *6* in a mouse writhing test (62). Further details are awaited with interest (does the activity reflect interaction at peripheral receptors, and is the effect naloxone-reversible?).

Physical Dependence

In a carefully devised study, Wei (69) examined seven analogues, i.e. [D-Ala^2,MePhe^4,Met(O)-ol^5], [D-Met^2,Pro-NH_2^5], [MeTyr^1,D-Ala^2,D-Met-NH_2^5], [MeTyr^1,D-Ala^2,D-Met^5], [D-Ala^2,Leu-NH_2^5], [D-Ala^2,D-Leu^5], and [D-Ala^2,Met-NH_2^5]enkephalin, all active after systemic administration, but of differing potencies, in common analgesic tests. After continuous three-day infusion of analogues (varying doses) into the brain (periaqueductal gray region) of rats by means of osmotic minipumps, a quantifiable withdrawal syndrome was produced in each case when an opiate antagonist was administered. There was good correlation between the ability of analogues to produce physical dependence and their antinociceptive activity. It is therefore tentatively concluded that analogues with potent analgesic activity will also be potent in producing physical dependence. Frederickson & Smithwick (63) observed little or no withdrawal symptoms after chronic administration of [D-Ala^2,MeMet-NH_2^5]-enkephalin to rats in increasing subcutaneous doses (10–160 mg/kg^{-1}), whereas morphine in similar doses produced a high level of dependence. However, the analgesic potency of this analogue is appreciably less than that of morphine, and its biological half-life was not determined. The results may not, therefore, contradict the above conclusion.

Respiratory Depression

There is very little published information relating to this classical opiate-like effect. Intravenous administration of Met- or Leu-enkephalin to pentobarbitone-anesthetized dogs caused brief depressions (10–20%) of respiratory amplitude which were compensated for by equally brief increases (30–60%) in respiratory rate (70). Small differences in the effects of Met- and Leu-enkephalin have been observed; given intracisternally, Met-, but not Leu-, enkephalin caused a reduction in respiration frequency in α-chloralose-anesthetized rats (71). In our own work (36) the effect on the respiratory rate of about 200 analogues (intravenously applied) was examined in conscious mice immediately before each animal was examined in the hot plate test. Most were approximately equi-respiratory depressant at equi-analgesic doses. There were, however, interesting exceptions, i.e. analogues which were relatively free from respiratory depressant activity at analgesic doses, e.g. Tyr-D-Ala-Gly-Phe(6H)-Leu-OH.

Cardiovascular Effects

An early report by Cowan et al (70) established that enkephalins can produce cardiovascular effects similar to those reported for narcotic drugs. These authors found that both Met- and Leu-enkephalin (3–100 $\mu g/kg^{-1}$) induced dose-dependent decreases in diastolic blood pressure and heart rate after intravenous administration to pentobarbitone-anesthetized dogs. The effects were naloxone-reversible. Since this is one of the few cases where biological activity can be demonstrated after intravenous injection of the natural enkephalins, it is curious that in subsequent published work these substances and analogues have only been administered centrally. Results following the latter route of administration have in fact contrasted markedly with those following the intravenous route (suggesting that at least part of the action of the enkephalins is mediated via peripheral receptors?). Laubie et al (72) administered relatively high doses (up to 500 μg) of Met-enkephalin intracisternally to chloralosed dogs and observed no change in blood pressure or heart rate. Bolme et al (73) also applied Met-enkephalin (10–300 μg) and Leu-enkephalin (1–100 μg) intracisternally, but to chloralosed rats, and observed a dose-related hypertension, which was not followed (as in the case of morphine and β-endorphin) by prolonged hypotension; Met-, but not Leu-, enkephalin also reduced the heart rate significantly. Feldberg & Wei (74) used chloralosed cats and found that Met-enkephalin (100–400 μg intraventricularly, or 400 μg intracisternally) was without effect. However, all three groups observed marked, long-lasting hypotension, bradycardia, and a reduction in splanchnic discharges following central administration of "stable" analogues. The analogues they used, and their additional observations, were as follows. In dogs, [D-Ala^2]-Met-enkephalin and its amide differed from morphine-like drugs in causing initial and transient hypertension and tachycardia (72). In rats, the situation was reversed; morphine (and β-endorphin) produced hypertension (lasting for 10–15 min) before the hypotension, while [D-Ala^2]-Met-enkephalin amide did not (73). In cats, [D-Ala^2,D-Leu^5]-enkephalin was less potent than morphine in producing bradycardia when administered intraventricularly (20–100 μg), but about 30 times more potent than morphine in lowering heart rate and blood pressure when administered intracisternally (the effect lasting over 2 hr) (74). The effect of systemic administration of the latter analogue was also examined; intravenously (30 $\mu g/kg^{-1}$) it lowered blood pressure for less than 10 min, while subcutaneously (100–500 $\mu g/kg^{-1}$) the effect lasted for 30 min (74).

No conclusions about S-A relations can be drawn from this small amount of published information. Our own unpublished work using, mainly, anesthetized rats shows that many analogues cause marked, but generally transient, falls in blood pressure after intravenous administration, and that

analogues which are inactive in the GPI assay do not show this effect. While, in general, molecular changes associated with increased analgesic potency in vivo also cause increased hypotension, this is not always so. Further exploration of analogues is clearly likely to provide interesting and unexpected results. Added stimulus for such work is provided by a recent finding that lowered blood pressure, following certain surgical procedures, is restored by naloxone, leading to speculation that enkephalins or endorphins may be involved in the physiological control of blood pressure, and are released by stress (75).

Release of Prolactin (PRL) and Growth Hormone (GH)

About 30 enkephalin-like peptides have so far been examined in unanesthetized, or steroid-primed and anesthetized rats. Ten, involving structural change that leads to inactivity in the GPI and MVD assays (e.g. the N-acetyl, D-Tyr[1], Ala[3], D-Phe[4], and Leu[4] analogues), were inactive after intraventricular administration (76, 77). The others all caused stimulation of PRL and, when measured, GH secretion, and in most cases the response was naloxone-reversible.[1] There is, however, disagreement about results in vitro. Rivier et al (79) report that Met-enkephalin, like morphine, is inactive in the cultured rat pituitary cell test, whereas Lien et al (80) report that the same compound causes significant release of PRL at 5 ng/ml. The reason for this discrepancy is unknown. In agreement with Rivier et al (79), more "stable" analogues are reported to be inactive in vitro (81, 82). In the in vivo tests the potency of analogues seems to relate to that found in analgesic tests. Thus, Met- and Leu-enkephalin are active after intraventricular injection only (83–85); Met-enkephalin amide (81), ethylamide (76), and [D-Ala[2]]-Met-enkephalin (76) are considerably more potent by this route, but inactive after subcutaneous injection; whereas the [D-Ala[2],Met-NH_2[5]] (81, 84, 76), [D-Ala[2],Leu-NH_2[5]] (81), and [D-Ala[2],D-Leu-OH] (86) analogues are active after subcutaneous injection. The [D-Met[2], Pro-NH_2[5]] analogue (compound *3* in Table 7) is the most potent releaser of PRL; it is five times more potent than morphine in PRL release and has half the potency of morphine in GH release (87). Similar selectivity toward PRL release is also seen with Met- and Leu-enkephalin; intraventricular doses of up to 10 μg (urethane-anesthetized rats) caused significant, dose-related elevation of plasma PRL levels, whereas plasma GH levels were unaffected (85). Interestingly, the [D-Ala[2],D-Leu-NH_2[5]] analogue is reported to have a different profile; it is said to be twice as potent (on a weight for weight basis) as morphine as a GH releaser, but about equipotent as a PRL releaser

[1]The release of PRL by Leu-enkephalin is claimed not to be antagonized by naloxone (78), but this report has not been confirmed.

(88). Since the change of Leu^5 to D-Leu^5 is thought to be associated with selectivity toward a second type (δ) of opiate receptor (49), the possibility that the PRL and GH releasing effects of enkephalins are mediated via different receptors needs investigation. Finally, one analogue (compound *1*, Table 7) has been investigated in man (82, 89); at doses of 0.1–1.2 mg, classical symptoms of morphine were not observed, but there was large, dose-related release of both PRL and GH. It thus seems that the threshold dose for PRL and GH release is appreciably less than that for other opiate-like effects.

Antidiarrheal Effects

In the mouse charcoal meal test, Met- and Leu-enkephalin (100–500 μg and 50–250 μg respectively, both intraventricularly) produced dose-related constipation. The effect, unlike that of morphine, was relatively difficult to antagonize by naloxone. The only published systematic study of S-A relationships is that of Miller et al (20). Of twelve peptides examined, all were active and Me-Tyr-D-Ala-Gly-Phe-D-Leu-NH_2 (ED_{50} 0.3 mg/kg^{-1}, subcutaneously) was the most potent. The list included one with a structure considerably removed from the enkephalins (Tyr-Ile-Asn-Met-Leu); the finding of activity in this compound suggests marked differences in the receptors involved for antidiarrheal and other opiate-like effects. Although described as antidiarrheal, the activity seen in charcoal meal tests is more accurately antimotility activity. In an unpublished study, J. H. Zavecz (Biomedical Research Dept., ICI Americas Inc.) examined 20 analogues also in the rat castor oil test, wherein the presence or absence of actual diarrhea was scored. Varying degrees of activity were seen by all analogues in both tests after intraperitoneal administration, and most were active orally. Some analogues were equally active in both tests, whereas others had weak antimotility activity but still marked antidiarrheal activity.

Other Effects

There was an early report (70) that Met-enkephalin (10–1000 μg, intraventricularly) induces hyperthermia in cats, whereas Leu-enkephalin (20–700 μg) induces hypothermia. Subsequent studies (e.g. 90), mainly in rats, indicate that "stable" analogues are similar to morphine in having biphasic effects on temperature; that is, they produce hyperthermia at low doses (lower than those necessary for eliciting analgesia), and hypothermia (most consistently in a cold environment) at higher doses.

The enkephalins, like β-endorphin and morphine, produce a wide range of behavioral effects, e.g. mood elevation, selective inhibition of conditioned avoidance, locomotor excitation or inhibition, extreme muscular rigidity, which are only partly explicable in terms of inhibition of dopamine systems.

Several analogues, e.g. [D-Met^2,Pro-NH$_2^5$]- (91) and [D-Ala^2,MePhe^4,Met-(O)-ol^5]- (92, 93) enkephalin, have been studied, but no significant differences in their profile of actions have yet emerged.

CONCLUSION

Study of S-A relationships of enkephalin-like peptides using isolated tissue and opiate receptor preparations has led to systemically active analogues with high potency in all in vivo tests for opiate-like activity. The achievements have practical significance in opening up new possibilities in drug therapy and have important implications in our understanding of the pharmacology of peptides in general. From previous work with peptide hormones, molecular modifications that promote oral absorption, give rise to increased metabolic stability, etc, had been identified. Work with the enkephalins has extended this knowledge considerably, and demonstrated convincingly the ability of many peptides to penetrate the central system. A particularly gratifying aspect of the work has been the involvement of large numbers of pharmacologists whose interest in peptides had previously been peripheral. As a result, classical pharmacological methods for analyzing modes of action and gaining information on receptors have been applied to peptides on an unprecedented scale. It is hoped that appetites have been whetted, and that systematic study of neglected topics (e.g. active transport of peptides across the blood-brain barrier, binding of peptides to plasma proteins) will now ensue.

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