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EFFECTS OF CHANGES IN THE STRUCTURE OF ENKEPHALINS AND OF NARCOTIC ANALGESIC DRUGS ON THEIR INTERACTIONS WITH μ- AND δ-RECEPTORS

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1 The activity pattern of analogues of the enkephalins was determined in four parallel assays, the inhibition of the electrically evoked contraction of the guinea-pig ileum and mouse vas deferens at 36°C and the inhibition of [^{3}H]-naltrexone and [^{3}H]-leucine-enkephalin binding at 0 to 4°C in homogenates of guinea-pig brain.

2 The activity pattern was best characterized by the ratio of the potency in the guinea-pig ileum to that in the mouse vas deferens (G.p.i./M.v.d.) and the ratio of the potency in inhibiting [^{3}H]-naltrexone binding to that in inhibiting [^{3}H]-leucine-enkephalin binding (Nal/Leu).

3 The enkephalins had low G.p.i./M.v.d. (0.02 to 0.09) and low Nal/Leu (0.05 to 0.18) ratios whereas the corresponding values for morphine were 7.0 and 7.5.

4 Analogues obtained by substituting D-Ala for Gly^2 and D-Met or D-Leu for L-Met^5 or L-Leu^5 showed only minor changes in G.p.i./M.v.d. (0.01 to 0.11) and in Nal/Leu (0.06 to 0.13) ratios.

5 Analogues in which resistance to enzymatic degradation was brought about by amidation of the C-terminal carboxylic group or methylation of the amino group of tyrosine or both modifications, had G.p.i./M.v.d. ratios of 1.2 to 5.5 and Nal/Leu ratios of 0.5 to 21. High values (2.1 and 3.4) were found for the potent antinociceptive analogue of Sandoz, Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol.

6 In the mouse vas deferens, some of the analogues with high G.p.i./M.v.d. and Nal/Leu ratios were tested for antagonism by naloxone and found to require less than the high concentration needed for the natural enkephalins. C57/BL mice, which have a lowered sensitivity to morphine but a normal response to peptides with low G.p.i./M.v.d. and Nal/Leu ratios, had a lowered sensitivity to analogues with high ratios.

7 In the alkaloid-like series of narcotic analgesic drugs, ketobemidone, levorphanol, methadone, etorphine and the antagonist Mr 2266 had lower Nal/Leu ratios (1.0 to 2.8) than morphine, normorphine, naloxone and naltrexone (8 to 12).

8 It would appear that compounds with low G.p.i./M.v.d. and Nal/Leu ratios interact mainly with δ-receptors in the brain and peripheral nervous system while compounds with high ratios interact mainly with μ-receptors. For antinociceptive action μ-receptors may be more important than δ-receptors.

Introduction

When Lord, Waterfield, Hughes & Kosterlitz (1977) first introduced the concept that the opioid peptides and narcotic analgesic drugs interact with different opiate receptors, they pointed out that modification of the enkephalin molecules may change their affinities for the naloxone and enkephalin binding sites. In this paper we have analyzed, by means of four parallel assays, the effects of such structural changes not only in opioid peptides but also in narcotic analgesic drugs. Some of the results have been reported to meetings of the U.S. Committee on Problems of Drug Dependence (Kosterlitz, Hughes, Leslie, Lord & Waterfield, 1977; Kosterlitz, McKnight, Waterfield, Gillan & Paterson, 1978).

Methods

Animals

Male guinea-pigs, 400 to 600 g, were used. The mice were of either the white TO inbred strain or the black C57/BL strain, weighing 25 to 30 g.

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Pharmacological assays

Two of the assays were based on pharmacological responses: the depression of the electrically evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum (Kosterlitz, Lydon & Watt, 1970) and of the mouse vas deferens (Hughes, Kosterlitz & Leslie, 1975).

The results were expressed as the IC_{50} values obtained from concentration-response curves. When peptides were assayed, methionine-enkephalin was used as internal standard because preparations varied considerably in sensitivity. For this reason, the results obtained for the various compounds were normalized in the following manner. The IC_{50} value obtained in an individual assay was multiplied by the fraction: (mean IC_{50} value of methionine-enkephalin obtained for the particular group of assays)/(IC_{50} of methionine-enkephalin found in the individual assay). The mean IC_{50} values of methionine-enkephalin for the different groups of assays are given in the legends to the tables.

Binding assays

The other two assays tested the inhibitory effects of the peptide analogues and the narcotic analgesics on the binding of [^{3}H]-naltrexone or of [^{3}H]-leucine-enkephalin by a modification of the method of Pert & Snyder (1973). The brain tissue, after removal of the cerebellum, was homogenized in Tris buffer (pH 7.4 at 0°C), centrifuged at 49,000 *g* for 10 min, the pellet resuspended in Tris buffer, incubated at 37°C for 45 min and centrifuged again. For the assay, 1.9 ml final homogenate without addition of Na^+ and corresponding to 19 mg of brain tissue, was used and the volume made up to 2.0 ml with solutions of the inhibitory cold ligand and of the labelled primary ligand. The mixture was incubated for 150 min at 0°C, filtered through Whatman GF/B glass filter discs and washed two or three times with 4 ml ice-cold Tris buffer solution. The material retained on the filters was solubilized with 1 ml Soluene, acidified with glacial acetic acid and after addition of Multisol II or Unisolve counted, at an efficiency of 35 to 40% on a Liquid Scintillation Spectrometer. Specific binding was the difference in the count when 50 nM of the antagonist Mr 2266 were present or absent. Its (+)-isomer, Mr 2267, did not inhibit binding in concentrations of up to 50 nM.

The results were obtained from the regression of log % inhibition of binding on log concentration of cold ligand and expressed as IC_{50}. The inhibition constant (K_I) was calculated from

$$K_I = \frac{IC_{50}}{1 + [L]/K_D}$$

where [L] is the concentration of the labelled ligand and K_D its equilibrium dissociation constant (Cheng & Prusoff, 1973). Cold methionine-enkephalin served as internal standard when cold peptides were used but no correction was made for variations of the IC_{50} values of methionine-enkephalin because it would not have reduced the coefficient of variation. For the high affinity binding sites, the K_D values at 0°C were 0.46 nM for [^{3}H]-naltrexone and 1.14 nM for [^{3}H]-leucine-enkephalin (Waterfield, Leslie, Lord & Kosterlitz, unpublished observation).

Primary labelled ligands

These were [^{3}H]-naltrexone (10.8 Ci/mmol, 0.4 nM), [^{3}H]-naloxone (4 Ci/mmol, 1 nM), both from the National Institute on Drug Abuse, and [^{3}H]-leucine-enkephalin (20 to 40 Ci/mmol, 0.86 nM; Radiochemical Centre, Amersham).

Drugs and peptides

The following drugs were used: etorphine hydrochloride (Reckitt & Colman); ketobemidone hydrochloride and (−)-α-5,9-diethyl-2-(3-furyl-methyl)-2′-hydroxy-6,7-benzomorphan as the free base (Mr 2266) (Dr H. Merz, Boehringer Sohn, Ingelheim); levorphanol tartrate (Roche Products); methadone hydrochloride and normorphine hydrochloride (Wellcome Laboratories); morphine hydrochloride (Macfarlan Smith); naloxone hydrochloride and naltrexone hydrochloride (Endo Laboratories); (±)-N-amyl-3-hydroxy-morphinan hydrochloride and N-amylnorketobemidone hydrochloride (Dr E. L. May).

All peptides were synthetic. Tyr-D-Ala-Gly-Phe-D-Met and Tyr-D-Ala-Gly-Phe-D-Leu were supplied by Dr S. Wilkinson (Wellcome Laboratories), samples of Tyr-D-Ala-Gly-Phe-L-Leu from Dr S. Wilkinson and Dr J. S. Morley (I.C.I.), Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol by Dr D. Roemer (Sandoz) and the other peptides by Dr B. A. Morgan (Reckitt & Colman).

Stock solutions of the peptides (1 or 2 mg/ml) were made in distilled water and kept in plastic tubes at −25°C. Stock solutions of the other compounds were made in distilled water, with addition of HCl when necessary. The concentrations are given in nM.

Results

Analogues retaining the activity pattern of the enkephalins

Relatively few analogues are known which possess the activity pattern of the naturally occurring enkephalins. Replacement of glycine in position 2 by D-alanine in leucine-enkephalin did not change the binding

BJP 68 2-1

Table 1 Changes in the inhibitory effects of methionine-enkephalin and leucine-enkephalin due to substitution of Gly[2] by D-Ala and Met[5] and Leu[5] by D-Met or D-Leu

Peptide	Inhibition of contractions (IC_{50}, nM)		Inhibition of binding in brain homogenates			
			[^3H]-naltrexone (0.4 nM)		[^3H]-leucine-enkephalin (0.86 nM)	
	Guinea-pig ileum	Mouse vas deferens	(IC_{50}, nM)	K_I (nM)	(IC_{50}, nM)	K_I (nM)
Tyr-Gly-Gly-Phe-L-Met	157 ± 17 (30)	14.7 ± 1.4 (28)	11.6 ± 0.70 (11)	6.2	1.93 ± 0.19 (12)	1.10
Tyr-D-Ala-Gly-Phe-L-Met	28.0 ± 1.7 (6)	2.96 ± 0.53 (7)	20.7 ± 2.8 (4)	12.1	1.60 ± 0.33 (3)	0.91
Tyr-D-Ala-Gly-Phe-D-Met	87.2 ± 2.3 (4)	2.50 ± 0.51 (5)	60.8 ± 5.2 (3)	32.5	3.54 ± 0.66 (3)	2.02
Tyr-Gly-Gly-Phe-L-Leu	455 ± 53 (13)	8.82 ± 1.18 (7)	46.6 ± 6.4 (3)	24.9	2.20 ± 0.40 (3)	1.26
Tyr-D-Ala-Gly-Phe-L-Leu	28.7 ± 1.9 (3)	1.63 ± 0.28 (4)	45.2 ± 10.8 (3)	24.2	3.57 ± 1.09 (3)	2.04
Tyr-D-Ala-Gly-Phe-D-Leu	47.8 ± 4.4 (4)	0.54 ± 0.09 (5)	55.6 ± 4.3 (3)	29.7	6.75 ± 1.65 (3)	3.86

The values are the means ± s.e. mean; the number of observations is given in parentheses. The mean values of methionine-enkephalin in the first horizontal line were obtained from their measurements as internal standard. In the guinea-pig ileum and the mouse vas deferens the individual values obtained for the other peptides were normalized by multiplying them by the ratio of the methionine-enkephalin values, mean IC_{50}/individual IC_{50}. For Tyr-D-Ala-Gly-Phe-L-Met [^{3}H]-naloxone (1 nM) was used instead of [^{3}H]-naltrexone.

Table 2 Changes in the relative agonist potencies of methionine-enkephalin and leucine-enkephalin due to substitution of Gly[2] by D-Ala and Met[5] or Leu[5] by D-Met or D-Leu

Peptide	Relative inhibition of contractions			Relative inhibition of binding in brain homogenates		
	Guinea-pig ileum	Mouse vas deferens	G.p.i./M.v.d.	[^3H]-naltrexone	[^3H]-leucine-enkephalin	Nal/Leu
Tyr-Gly-Gly-Phe-L-Met	0.094	1.0	0.094	0.18	1.0	0.18
Tyr-D-Ala-Gly-Phe-L-Met	0.53	5.0	0.106	0.091	1.21	0.08
Tyr-D-Ala-Gly-Phe-D-Met	0.17	5.9	0.029	0.034	0.54	0.06
Tyr-Gly-Gly-Phe-L-Leu	0.032	1.7	0.019	0.044	0.87	0.05
Tyr-D-Ala-Gly-Phe-L-Leu	0.51	9.0	0.057	0.045	0.54	0.08
Tyr-D-Ala-Gly-Phe-D-Leu	0.31	27.2	0.011	0.037	0.28	0.13

For the measurement of the inhibition in the guinea-pig ileum and mouse vas deferens, the IC_{50} value of methionine-enkephalin in the mouse vas deferens was taken to be 1; for the measurement of inhibition in the brain homogenates, the K_I value of methionine-enkephalin against [^{3}H]-leucine-enkephalin binding was taken to be 1.

characteristics significantly although the potency in the mouse vas deferens increased 5 fold and in the guinea-pig ileum over 15 fold (Tables 1 and 2). The effects of a similar substitution in methionine-enkephalin did not affect the inhibition of [^{3}H]-leucine-enkephalin binding but halved that of [^{3}H]-naltrexone binding; the increase in activity was of the same order in the guinea-pig ileum and mouse vas deferens, about 5 to 6 fold. The increased potency in the pharmacological assays without corresponding potency changes in the binding assays is probably due mainly to a decreased degradation in the biophase of the tissues.

When, in addition to the changes in the second amino acid residue, the C-terminal amino acid was replaced by its D-isomer, further important alterations in the activity pattern were found. In the methionine series this substitution reduced the affinity to both the [^{3}H]-naltrexone and the [^{3}H]-leucine-enkephalin binding sites by 55 to 60% (Tables 1 and 2). The potency in the guinea-pig ileum decreased by about 70%, a value which corresponds approximately with the decrease in affinity to both binding sites. In the mouse vas deferens, there was no change in the potency, a fact which appears to indicate that there has been a 3 to 4 fold shift in relative potency in favour of this tissue. Introduction of D-leucine in D-Ala^2-L-Leu^5-enkephalin did not cause any significant change in the affinity to the two binding sites but increased the activity in the mouse vas deferens and reduced it in the guinea-pig ileum, so that there was a 5 fold shift in potency towards the mouse vas deferens without corresponding affinity changes indicated by the binding assays.

The ratios of the inhibitory potency in the guinea-pig ileum to that in the mouse vas deferens (G.p.i./M.v.d.) and of the potency in inhibiting [^{3}H]-naltrexone binding to that in inhibiting [^{3}H]-leucine-enkephalin binding (Nal/Leu) had low values which are characteristic for the analogues described so far. They ranged from 0.01 to 0.11 for G.p.i./M.v.d. and 0.06 to 0.13 for Nal/Leu, compared with corresponding values of 0.02 and 0.09 and 0.05 and 0.18, respectively, for natural leucine- and methionine-enkephalin (Table 2).

Analogues with an activity pattern more similar to morphine

It has been shown previously that the free carboxyl-group at the C-terminal of the enkephalins is important for the retention of their natural activity pattern. Decarboxylation of Tyr-D-Ala-Gly-Phe-Leu, which leads to an isoamyl amide of the tetrapeptide Tyr-D-Ala-Gly-Phe, increased the inhibition of [^{3}H]-naltrexone binding associated with some decrease of inhibition of [^{3}H]-leucine-enkephalin binding. There was no change of potency in the guinea-pig ileum but a loss in activity of more than 90% in the mouse vas deferens (Tables 3 and 4). If now a methyl group was attached to the N-atom of tyrosine, the inhibition of the [^{3}H]-leucine-enkephalin binding was decreased by almost 85% and that of [^{3}H]naltrexone binding by 72%. The potency in the mouse vas deferens was decreased by a further 75% and in the quinea-pig ileum by 40%. Compared with Tyr-D-Ala-Gly-Phe-Leu, NCH$_3$Tyr-D-Ala-Gly-Phe isoamyl amide had about the same potency in inhibiting [^{3}H]-naltrexone binding, 7.4% of that in inhibiting [^{3}H]-leucine-enkephalin binding, about 70% of the potency in the guinea-pig ileum and 1.5% of the potency in the mouse vas deferens. The extent of the change in activity pattern is best seen in the increase of the ratio G.p.i./M.v.d. from 0.08 to 3.8 and that of Nal/Leu from 0.08 to 1.2.

The free tetrapeptide, Tyr-D-Ala-Gly-Phe, had only 10% of the activity of its isoamyl amide but the activity patterns of the two compounds were similar with a G.p.i./M.v.d. ratio of about 1.5 and a Nal/Leu ratio of about 0.6 (Tables 3 and 4). They differed from corresponding values of the natural enkephalins (Table 2). When the tetrapeptide was decarboxylated to give the phenylethyl amide of the tripeptide Tyr-D-Ala-Gly, its potency in inhibiting [^{3}H]-naltrexone binding increased without major changes in the other parameters. The effect of introducing a methyl group at the N-atom of tyrosine was a reduction of potency in the mouse vas deferens. In addition, the potency for inhibition of [^{3}H]-leucine-enkephalin binding was reduced by 85% with a lesser decrease in the inhibition of [^{3}H]-naltrexone binding. The ratios G.p.i./M.v.d. and Nal/Leu were now of an order similar to that found for morphine (Table 5).

Another analogue which had a morphine-like activity pattern was Tyr-D-Ala^2-Gly-NCH$_3$Phe-Met(O)-ol (Roemer, Buescher, Hill, Pless, Bauer, Cardinaux, Closse, Hauser & Huguenin, 1977). Compared with morphine, this peptide was 10 times more potent in the guinea-pig ileum, 30 times more potent in the mouse vas deferens, 3 times more potent as inhibitor of [^{3}H]-naltrexone binding and about 7 times more potent as inhibitor of [^{3}H]-leucine-enkephalin binding (Table 5). Compared with D-Ala^2-methionine-enkephalin, it was 4 times more potent in the guinea-pig ileum, had 20% of the potency in the mouse vas deferens, was about 4 times more potent as inhibitor of [^{3}H]-naltrexone binding but had only about 10% of the potency for inhibition of [^{3}H]-leucine-enkephalin binding. The ratio of G.p.i./M.v.d. was 2.1 and that of Nal/Leu 3.4; these findings indicate that the activity pattern of this analogue was more like that of morphine than of D-Ala^2-methionine-enkephalin or the natural methionine-enkephalin.

Table 3 Changes in the inhibitory effects of Tyr-D-Ala-Gly-Phe-Leu and Tyr-D-Ala-Gly-Phe due to decarboxylation of the C-terminal amino acid residue and introduction of a CH_3-group at the N of Tyr

Peptide	*Inhibition of contractions (IC_{50}, nM)*		*Inhibition of binding in brain homogenates*			
			[^3H]-naltrexone (0.4 nM)		*[^3H]-leucine-enkephalin (0.86 nM)*	
	Guinea-pig ileum	*Mouse vas deferens*	*(IC_{50}, nM)*	*K_I (nM)*	*(IC_{50}, nM)*	*K_I (nM)*
Tyr-D-Ala-Gly-Phe-Leu	24.9 ± 1.6 (3)	2.10 ± 0.37 (4)	45.2 ± 10.8 (3)	24.2	3.57 ± 1.1 (3)	2.04
Tyr-D-Ala-Gly-PheNH$(CH_2)_2$CH$(CH_3)_2$	21.9 ± 4.0 (9)	34.2 ± 12.4 (3)	12.4 ± 1.1 (3)	6.6	6.27 ± 0.75 (3)	3.58
NCH_3-Tyr-D-Ala-Gly-PheNH$(CH_2)_2$CH$(CH_3)_2$	36.7 ± 4.0 (4)	137 ± 29.0 (4)	42.6 ± 2.8 (3)	22.8	48.3 ± 6.7 (3)	27.6
Tyr-D-Ala-Gly-Phe	206 ± 14.7 (4)	315 ± 74.1 (3)	137 ± 26.9 (3)	73.3	84.0 ± 17.0 (3)	48.0
Tyr-D-Ala-GlyNH$(CH_2)_2$Ph	155 ± 35.0 (4)	431 ± 79.1 (5)	24.1 ± 2.3 (3)	12.9	141 ± 15.7 (3)	80.6
NCH_3Tyr-D-Ala-Gly-NH$(CH_2)_2$Ph	272 ± 77.8 (3)	1474 ± 251 (6)	52.9 ± 9.8 (3)	28.3	1032 ± 258 (3)	590

The values are the means ± s.e. mean; the number of observations is given in parentheses. The values obtained in the guinea-pig ileum and mouse vas deferens were normalized as described in Table 1. The mean IC_{50} values of methionine-enkephalin were 136.4 ± 13.4 (n = 27) in the guinea-pig ileum, 18.9 ± 2.0 (n = 25) in the mouse vas deferens, 10.7 ± 1.1 (n = 9) for inhibition of [^{3}H]-naltrexone and 1.84 ± 0.22 (n = 9) for [^{3}H]-leucine-enkephalin binding.

Table 4 Changes in the relative potencies of Tyr-D-Ala-Gly-Phe-Leu and Tyr-D-Ala-Gly-Phe due to decarboxylation of the C-terminal amino acid residue and introduction of a CH_3-group at the N of Tyr

Peptide	*Relative inhibition of contractions*			*Relative inhibition of binding in brain homogenates*		
	Guinea-pig ileum	*Mouse vas deferens*	*G.p.i./M.v.d.*	*[^3H]-naltrexone*	*[^3H]-leucine-enkephalin*	*Nal/Leu*
Tyr-D-Ala-Gly-Phe-Leu	0.084	1	0.084	0.084	1	0.084
Tyr-D-Ala-Gly-PheNH$(CH_2)_2$CH$(CH_3)_2$	0.096	0.061	1.57	0.308	0.570	0.54
NCH_3-Tyr-D-Ala-Gly-PheNH$(CH_2)_2$CH$(CH_3)_2$	0.057	0.015	3.80	0.089	0.074	1.20
Tyr-D-Ala-Gly-Phe	0.010	0.0067	1.49	0.028	0.043	0.65
Tyr-D-Ala-GlyNH$(CH_2)_2$Ph	0.014	0.0049	2.86	0.158	0.025	6.3
NCH_3Tyr-D-Ala-Gly-NH$(CH_2)_2$Ph	0.0077	0.0014	5.5	0.072	0.0035	20.6

For the measurement of the inhibition in the guinea-pig ileum and mouse vas deferens, the IC_{50} value of Tyr-D-Ala-Gly-Phe-Leu in the mouse vas deferens was taken to be 1; for the measurement of inhibition of binding in the brain homogenates, the K_I value of Tyr-D-Ala-Gly-Phe-Leu against [^{3}H]-leucine-enkephalin was taken to be 1.

Table 5 Comparison of the relative potencies of Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol with methionine-enkephalin, its D-Ala^2-analogue and morphine

Compound	Relative inhibition of contractions			Relative inhibition of binding in brain homogenates	
	Guinea-pig ileum	Mouse vas deferens	G.p.i./M.v.d.	[^{3}H]-naltrexone	[^{3}H]-leucine-enkephalin Nal/Leu
Tyr-Gly-Gly-Phe-Met	0.094	1.0	0.09	0.18	1.0 0.18
Tyr-D-Ala-Gly-Phe-Met	0.53	5.0	0.11	0.091	1.2 0.08
Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol	2.0	0.94	2.1	0.22	0.064 3.4
Morphine	0.21	0.030	7.0	0.067	0.009 7.5

The mean IC_{50} values ($n = 4$) of Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol were 7.8 ± 1.4 nM (mean methionine-enkephalin = 167 ± 20 nM) in the guinea-pig ileum and 9.3 ± 2.3 nM (mean methionine-enkephalin = 8.7 ± 1.2 nM) in the mouse vas deferens. After adjustment to the methionine-enkephalin values in Table 1, the mean IC_{50} values of the analogue became 7.2 and 15.7 nM, respectively. The mean IC_{50} value ($n = 4$) for inhibition of [^{3}H]-naltrexone (0.4 nM) binding was 9.4 ± 1.4 nM and for that of [^{3}H]-leucine-enkephalin (0.86 nM) binding 30.1 ± 3.8 nM, giving K_i values of 5.0 and 17.2 nM, respectively. The relative potencies were calculated as described in Table 2, the K_i of methionine-enkephalin, 1.10 nM, being the standard of reference.

Further confirmation of this view was obtained by testing some of these peptides on the vasa deferentia of two strains of mice, one of which (C57/BL) was considerably less sensitive to the inhibitory action of morphine than the other (TO); in contrast, there was no difference between the two strains in their sensitivity to enkephalins (Waterfield, Lord, Hughes & Kosterlitz, 1978). In two sets of experiments (Table 6), it was found that Tyr-D-Ala-GlyNH$(CH_2)_2$Ph and NCH_3Tyr-D-Ala-GlyNH$(CH_2)_2$Ph were about 3 times, and normorphine 5 times, more potent in TO mice than in C57/BL mice. In contrast, methionine-enkephalin was almost twice as potent in the C57/BL as in the TO mice. Thus, these two enkephalin analogues that had acquired a morphine-like activity pattern in the four parallel assays (Table 4) showed the expected potency difference in the two strains of mice.

Effectiveness of naloxone as antagonist

It has been shown previously (Lord *et al.*, 1977) that, whereas in the guinea-pig ileum naloxone was about equally effective against normorphine and the opioid peptides, about 10 times more naloxone was required in the mouse vas deferens for the antagonism of the opioid peptides than for that of normorphine. It was therefore of interest to determine the effectiveness of naloxone in antagonizing enkephalin analogues which had acquired an activity pattern more like that of morphine than that of enkephalin. It was found that, whereas the K_e values of naloxone against analogues with an enkephalin-like activity pattern varied between 20 and 30 nM as compared with a value of about 2 nM against normorphine, the K_e values against Tyr-D-Ala-GlyNH$(CH_2)_2$Ph and Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol were only 4 to 6 nM (Table 7). This finding is in agreement with the view that the activity pattern of these two analogues had become more morphine-like.

Activity patterns of narcotic analgesic drugs

It was of interest to examine whether analgesic compounds of the phenylpiperidine, benzomorphan, morphine and oripavine series showed variations in their affinities for the naltrexone and leucine-enkephalin binding sites which were similar to those found for opioid peptides.

Some of these compounds were found to have a greater affinity for the enkephalin binding sites than others (Table 8). When the ratios of Nal/Leu were determined, two groups could be distinguished. The first group had ratios between 7.7 and 12 and included morphine and normorphine as agonists and naloxone and naltrexone as antagonists. The second group had a greater relative affinity for the [^{3}H]-leucine-enkephalin binding site as shown by the smaller

ratios of between 1 and 2.8 and consisted of ketobemidone, levorphanol, methadone, etorphine and the N-amyl homologues of ketobemidone and levorphanol as agonists and (−)-α-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan (Mr 2266) as antagonist. Due to its increased affinity for the [^{3}H]-leucine-enkephalin binding site, the N-amyl homologue of ketobemidone had a smaller ratio than its parent substance; no such difference was found for the N-amyl homologue of levorphanol.

Discussion

The results presented in this paper confirm and extend the concept developed by Lord *et al.* (1977) that there are in the guinea-pig brain at least two types of opiate binding sites. [^{3}H]-naltrexone or [^{3}H]-naloxone binds preferentially to one of these sites and [^{3}H]-leucine-enkephalin to the other. The [^{3}H]-naltrexone binding site appears to correspond to the μ-receptors described in the chronic spinal dog (Martin, Eades, Thompson, Huppler & Gilbert, 1976) and in the myenteric plexus of the guinea-pig ileum (Lord *et al.*, 1977). In contrast, the [^{3}H]-leucine-enkephalin binding site in the brain corresponds more to the δ-receptors postulated to be the main receptors in the mouse vas deferens (Lord *et al.*, 1977). There is no evidence that methionine-enkephalin binds to a site different from the leucine-enkephalin binding site (Waterfield, Leslie, Lord, Ling & Kosterlitz, 1979.

Since the biological half-time of the two naturally occurring enkephalins is very short (e.g. Hughes, 1975; Hambrook, Morgan, Rance & Smith, 1976), many attempts have been made to design stable analogues with strong antinociceptive activity. It is therefore important to know which alterations in the molecule are permissible without concomitant changes in the pattern of pharmacological activity. Such changes

Table 6 The inhibitory actions (IC_{50}, nM) of normorphine, methionine-enkephalin and two analogues on the contractions of the vas deferens of two strains of mice

Compound	*TO mice*	*C57/BL mice*
Normorphine	363 ± 125 (5)	1888 ± 534 (6)
Methionine-enkephalin	24.7 ± 3.6 (5)	13.7 ± 2.3 (6)
Tyr-D-Ala-GlyNH$(CH_2)_2$Ph	595 ± 195 (4)	2208 ± 790 (6)
Normorphine	—	1389 ± 249 (7)
Methionine-enkephalin	21.4 ± 6.0 (7)	13.6 ± 2.2 (7)
NCH_3Tyr-D-Ala-GlyNH$(CH_2)_2$Ph	1054 ± 288 (7)	2941 ± 545 (7)

The values are the means ± s.e. mean; the number of observations is given in parentheses. In C57/BL mice, the mean IC_{50} values obtained in 17 paired experiments were for normorphine 2027 ± 411 nM and for methionine-enkephalin 13.2 ± 1.4 nM. The effectiveness of the antagonism of naloxone against the two analogues (from above downwards) gave K_e values of 4.09 ± 1.90 nM (3) and 3.63 ± 0.84 nM (4) in the TO mice and 3.21 ± 0.88 (3) and 6.81 ± 0.93 nM in the C57/BL mice.

Table 7 The effectiveness of naloxone (K_e, nM) in antagonizing the agonist actions of analogues of the enkephalins in the guinea-pig ileum and the mouse vas deferens

Compound	*Guinea-pig ileum*	*Mouse vas deferens*
Tyr-Gly-Gly-Phe-L-Met	2.45 ± 0.26 (4)	22.6 ± 0.5 (4)
Tyr-D-Ala-Gly-Phe-L-Met	2.55 ± 0.26 (4)	22.4 ± 2.7 (4)
Tyr-D-Ala-Gly-Phe-D-Met	2.19, 2.62	26.2 ± 5.4 (4)
Tyr-D-Ala-Gly-Phe-D-Leu	1.67, 2.19	31.9 32.3
Tyr-D-Ala-Gly-PheNH$(CH_2)_2CH(CH_3)_2$	1.74, 1.76	19.5, 25.2
Tyr-D-Ala-GlyNH$(CH_2)_2$Ph	1.53, 2.09	4.1 ± 1.9 (3)
Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol	3.76 ± 0.31(3)	5.7 ± 1.2(5)

The values are the means ± s.e. mean; when the number of observations is more than two, it is given in parentheses.

Table 8 The inhibitory effects of narcotic analgesics and their antagonists on the binding of [^{3}H]-naltrexone (Nal, 0.4 nM) and [^{3}H]-leucine-enkephalin (Leu, 0.86 nM) in homogenates of guinea-pig brain

Compound	Inhibition of binding (IC_{50}, nM)		Inhibition constants (K_i, nM)		Relative potencies		
	Nal	*Leu*	*Nal*	*Leu*	*Nal*	*Leu*	*Nal/Leu*
Morphine	30.7 ± 2.0 (8)	220 ± 34 (9)	16.4	126	1	0.13	7.7
Normorphine	39.5 ± 7.3 (3)	440 ± 49 (3)	21.1	252	0.78	0.065	12
Naloxone	2.26 ± 0.34 (3)	17,27	1.21	12.6	14	1.3	11
Naltrexone	0.70 ± 0.09 (7)	6.14 ± 0.67 (7)	0.37	3.51	44	4.7	9.4
Ketobemidone	38.9 ± 7.7 (3)	104 ± 2.3 (4)	20.8	59.4	0.79	0.28	2.8
N-amylnor-ketobemidone	29.6 ± 5.7 (3)	24.1 ± 2.7 (4)	15.8	13.8	1.04	1.19	0.9
(−)-N-methyl-3-hydroxymorphinan	2.31 ± 0.18 (3)	5.8 ± 0.64 (3)	1.24	3.31	13	5.0	2.6
(±)-N-amyl-3-hydroxymorphinan	3.67 ± 0.25 (3)	9.6 ± 1.23 (4)	1.96	5.49	8.4	3.0	2.8
Methadone	25.8 ± 2.4 (3)	24.7 ± 2.4 (3)	13.8	14.1	1.2	1.2	1.0
Etorphine	1.71 ± 0.32 (6)	2.81 ± 0.31 (5)	0.91	1.61	18	10	1.8
Mr 2266	0.75 ± 0.07 (5)	1.02 ± 0.16 (5)	0.40	0.58	41	28	1.5

The values are the means ± s.e. mean; the number of observations is given in parentheses. The relative potencies are expressed as $^1/K_i$, the inhibition constant of morphine against [^{3}H]-naltrexone being 1.

are best assessed by determining the ratio of potency in the guinea-pig ileum to that in the mouse vas deferens, G.p.i./M.v.d., and the ratio of inhibition of [^{3}H]-naltrexone binding to that of [^{3}H]-leucine-enkephalin binding, Nal/Leu.

It is characteristic for analogues which retain the activity pattern of the natural enkephalins that the ratios G.p.i./M.v.d. (0.01 to 0.1) and Nal/Leu (0.05 to 0.18) remain low. Examples of such analogues are enkephalins in which glycine in position 2 has been replaced by D-alanine and the terminal amino acid by its D-isomer. This relative constancy of these ratios does not exclude major changes in overall potency. For instance, in the pharmacological but not in the binding assays, the D-Ala^2-analogues were 5 to 14 times more potent than the natural Gly^2-enkephalins. Since the binding assays were performed at about 0°C and the pharmacological assays at about 36°C, metabolic effects will occur almost exclusively in the latter. Thus, in the biophase of the guinea-pig ileum and the mouse vas deferens, there will be considerable degradation of the natural enkephalins but not, or at least to a much lesser extent, of the analogues with D-alanine in position 2 which are protected aginst the action of aminopeptidases. From the data presented it is likely that the tritiated D-Ala^2-D-Met^5-enkephalin or D-Ala^2-D-Leu^5-enkephalin may be the ligands most suitable for the investigation of the enkephalin binding sites since they are sufficiently stable for binding assays to be performed at 25°C (Gillan, Kosterlitz & Paterson, 1979).

Different results were obtained when protection against enzymatic degradation was achieved by amidation of the C-terminal amino acid and methylation of the amino group of the N-terminal tyrosine. When both changes were brought about simultaneously, the ratio G.p.i./M.v.d. increased from 0.09 to 1.2 and Nal/Leu from 0.18 to 2.7 (Lord *et al.*, 1977). Similar changes were observed when the free carboxyl group of leucine was removed in D-Ala^2-L-Leu^5-enkephalin, leading to Tyr-D-Ala-Gly-PheNH$(CH_2)_2$CH$(CH_3)_2$. Such decarboxylation increased the ratio G.p.i./M.v.d. from 0.08 to 1.6 and Nal/Leu from 0.08 to 0.54. When the N-atom was methylated at the same time the ratios became 3.8 and 1.2, respectively. These findings confirm the view that the free carboxylic group of the C-terminal amino acid and the primary amino group of tyrosine are essential for the maintenance of an enkephalin-like pattern of activity.

The tetrapeptide, Tyr-D-Ala-Gly-Phe, has less overall activity than D-Ala^2-L-Leu^5-enkephalin. Its G.p.i./M.v.d. and Nal/Leu ratios are higher than those of the pentapeptide, demonstrating the importance of Leu^5 or Met^5 for the pharmacological pattern of the enkephalins as well as their potencies. Decarboxylation of the tetrapeptide and subsequent methylation of the amino group of tyrosine had effects on the

activity pattern similar to those obtained when corresponding changes were effected in the pentapeptide.

The analogue Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol (Sandoz) is of particular interest because it is a highly potent antinociceptive agent in rodents and is active even after oral administration (Roemer *et al.*, 1977). Its ratios G.p.i./M.v.d. of 2.1 and Nal/Leu of 3.4 were about midway between those of the natural enkephalins and of morphine; in all assays, its activity was much greater than that of morphine. Compared with narcotic analgesic drugs the Nal/Leu ratio was lower than that of morphine but higher than that of methadone or levorphanol.

Another enkephalin analogue, which after intravenous injection has a potency similar to that of the Sandoz analogue, is Tyr-D-Met-Gly-Phe-Pro amide (Székely, Rónai, Dunai-Kovács, Miglécz, Berzétei, Bajusz & Gráf, 1977). These authors found that this compound had a G.p.i./M.v.d. ratio of 1; we obtained similar results and in addition a Nal/Leu ratio of 1.8 (Kosterlitz *et al.*, 1978).

The low effectiveness of naloxone against the action of the natural opioid peptides on the mouse vas deferens supports the view that the peptides interact in this organ preferentially with a receptor population (δ-receptors) different from the μ-receptors with which the classical opiates interact. However, it should be noted that in the guinea-pig ileum the opioid peptides appear to interact preferentially with μ-receptors since their action is as readily antagonized by naloxone as that of morphine (Lord *et al.*, 1977; present results). This concept is supported by the observations (Table 7) that enkephalin analogues which have an activity pattern more like that of morphine than that of natural enkephalin, require, in the mouse vas deferens, less naloxone for antagonism than the natural opioid peptides. Similar results have been obtained by Rónai, Berzétei & Bajusz (1977) who showed that D-Met^2-Pro^5 amide was more readily antagonized by naltrexone than is methionine-enkephalin.

The third type of evidence for the view that some of the enkephalin analogues are morphine-like was obtained in experiments on C57/BL mice which are less sensitive to morphine than TO mice. In contrast to the natural enkephalins, the morphine-like analogues had a lower potency in the C57/BL mice than in the TO mice.

The analogues which have the highest relative affinity for the enkephalin binding site or the δ-receptor are the D-Ala^2-D-Met^5- and D-Ala^2-D-Leu^5-enkephalins since they have the lowest G.p.i./M.v.d. and Nal/Leu ratios. The highest ratios were found for the Sandoz analogue of high overall potency and the decarboxylated analogues of low potency. Both D-Ala^2-D-Leu^5-enkephalin and the Sandoz analogue have antinociceptive actions in rodents (Baxter, Goff, Miller & Saunders, 1977; Roemer *et al.*, 1977) but J. Bläsig & A. Herz (personal communication) have found that, after intraventricular administration, the Sandoz analogue, with a K_I against [^{3}H]-leucine-enkephalin binding of 17.2 nM, is about 100 times more potent as an antinociceptive agent than D-Ala^2-D-Leu^5-enkephalin which against the same ligand has a K_I of 3.9 nM. Since the rank order of the K_I values against [^{3}H]-naltrexone binding was reversed, namely 5 nM for the Sandoz analogue and 30 nM for the D-Ala^2-D-Leu^5-analogue, it would appear that the μ-receptor is of particular importance for antinociception, a view which has been put forward by Law & Loh (1978) and was also developed at a recent symposium on endorphins (Rónai & Berzétei, 1978; Kosterlitz, 1978). It may be of physiological importance that β-endorphin which is the most potent antinociceptive natural opioid peptide, has a high affinity not only for the δ-receptor but also for the μ-receptor (Lord *et al.*, 1977; Simantov, Childers & Snyder, 1978; Law & Loh, 1978).

Finally, the fact that the Nal/Leu ratios of the narcotic analgesics so far examined show quite considerable variations may be of importance for some of their pharmacological effects and it may be possible to synthesize novel types of alkaloid-like narcotic analgesics with patterns of activity similar to those of some of the enkephalin analogues.

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