OPIATE RECEPTOR AFFINITY OF PEPTIDES RELATED TO LEU-ENKEPHALIN

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SUMMARY - Several analogs of Leu-enkephalin were synthesized by the standard solid phase procedure in order to investigate structural requirements for binding to opiate receptors. Decisive features for receptor interaction seem to be the presence and location of the aromatic side chains of the tyrosine and phenylalanine residues. The terminal amino and carboxyl groups do not contribute significantly to binding affinity.

Hughes et al. (1) recently succeeded in characterizing and synthesizing two pentapeptides, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, with morphine-like activity. Our communication reports the synthesis of the latter compound and of several analogs. All analogs with two exceptions are characterized by the deletion, addition or substitution of one amino acid. The peptides were analyzed for their affinity for opiate receptors, as measured by competition with the opioid agonist, dihydromorphine. The affinities were compared with those of Tyr-Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gly-Lys and of morphine.

MATERIALS AND METHODS

All the peptides were prepared by solid-phase synthesis (2,3) and purified by gel filtration. Purity was established by thin-layer chromatography and amino-acid analysis after hydrolysis.

Synthesis of peptides. Boc-amino acids were exclusively used for the synthesis of all peptides. The side-chain of tyrosine was protected by a 2,6-dichlorobenzyl group (4). Chloromethylated copoly (styrene - 1 % divinylbenzene) beads (Bio-Beads SX-1, 200-400 mesh, from Bio-Rad Laboratories) served for attachment of the C-terminal amino acid. This resin was then loaded into the reaction vessel of a Beckman model 990 Peptide Synthesizer. The instrument was run essentially according to the recommendation of the manufacturer. 50 % trifluoroacetic acid was used for removal of Boc-groups. The coupling step was always repeated once before proceeding to the next amino acid. After

completed synthesis, peptides were detached from the resin with liquid HF (5) in the presence of anisole, extracted with 10 % acetic acid and the solutions lyophilized. The crude products were dissolved in a small quantity of 50 % acetic acid and passed through a Sephadex G-15 column (1.4 x 140 cm). In this solvent mixture all peptides gave symmetrical major peaks (UV 254 or 280 nm) together with varying quantities of well resolved but unidentified impurities. The lyophilized peptides were checked for purity by TLC on silica gel plates in three solvent systems. Spots were visualized in UV and by ninhydrin. Most peptides were estimated to be at least 97-98 % pure (peptides V, X and XI about 95 % pure, peptide XV 90 % pure) apart from ninhydrin negative material.

An aliquot of each peptide was hydrolyzed in 6 N HCl, containing 10 % phenol, for 24 h at 110°C in a sealed tube and then analyzed on an updated Beckman Spinco Amino Acid Analyzer. The amino acid ratios of all analogs were within 4 % of the theoretical values.[1] The true peptide content of all preparations was determined simultaneously.

100 μmole of peptide resin gave on average 25 mg of gel filtered peptide. We deliberately sacrificed higher yields in order to achieve the best possible purity of the products.

Receptor binding of peptides. Details of the procedure for receptor preparations and analysis of binding affinity have been described (6). Briefly, a synaptic plasma membrane (SPM) fraction from rat brain (corresponding to 0.4 mg protein) is incubated in 0.4 ml of HEPES buffer of pH 7.4, containing 0.4×10^{-9} M dihydromorphine-^{3}H and test peptides for 40 min at 25°C. Incubation is terminated by centrifugation in a Microfuge (Beckman) in the cold for 5 min. The SPM pellets are digested with Soluene (Packard) and the content of radioactivity is measured. Logarithmic dilutions of test peptides were added to the incubation solution and their inhibitory potencies were determined. Every run included both samples without peptides (controls) and samples with an excess, 10^{-6} M, of non-radioactive dihydromorphine to produce blank values. Blank values were substracted from all experimental values and the effect of a peptide in displacing the radioactive dihydromorphine was expressed in per cent of the control. Values of half-maximum inhibition were obtained graphically in semi-logarithmic plots. Each substance was tested at least 3 times at 3 or more concentrations.

RESULTS AND DISCUSSION

Receptor affinities for the synthetic peptides are summarized in Table 1. All active compounds gave essentially parallel dose-inhibition curves which were also parallel to those obtained with unlabelled dihydromorphine as competitor. This suggests a competitive interaction. The parent compound, Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) has a considerable affinity for the receptors, being of about the same magnitude as morphine.

It is also evident that the structure of the parent molecule is rather

[1] Peptide XV, β-LPH 61-69, showed a tyrosine value 15 % below theoretical. The other amino acids were within 6 % of theoretical values.

TABLE 1

Relative molar affinities of various peptides for opiate receptors of synaptic plasma membranes.

Substance	Relative receptor affinity
Tyr-Gly-Gly-Phe-Leu (I)	100
Tyr-Gly-Gly-Phe-Met (II, β-LPH$_{61-65}$)	300
Tyr-Tyr-Gly-Gly-Phe-Leu (III)	100
Tyr-Gly-Gly-Phe-Leu-Leu (IV)	60
Tyr-Gly-Gly-Gly-Phe-Leu (V)	15
Tyr-Gly-Phe-Leu (VI)	<1
Tyr-Gly-Gly-Phe (VII)	7
Gly-Gly-Phe-Leu (VIII)	<1
diJ-Tyr-Gly-Gly-Phe-Leu (IX)	<1
Tyr-Gly-Gly-Tyr-Leu (X)	3
Phe-Gly-Gly-Phe-Leu (XI)	<3
Tyr-Ala-Gly-Phe-Leu (XII)	3
Tyr-Gly-Ala-Phe-Leu (XIII)	6
Tyr-Ala-Ala-Phe-Leu (XIV)	<1
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys (XV, β-LPH$_{61-69}$)	300
Morphine [1]	800

[1] $K_D = 5 \cdot 10^{-9}$ M in the present system

critical. Increases in chain length at both the N-terminal and the C-terminal ends are entirely permissible. It is particularly surprising that the amino group of Tyr is not essential, since an amino nitrogen (not peptide or amide nitrogen) protonated at physiological pH has been considered to be essential for the interaction of an opioid with the receptor (7). The increase in length of the molecule with one Gly reduced activity only slightly while de-

letion of one Gly or either of the terminal amino acids (Tyr or Leu) gives inactive compounds. Since no systematic modifications at the C-terminus were made, it is not known how critical this function is. The Met-analog is identical to sequence 61-65 in β-LPH. The longer sequence 61-69 was equally active, indicating that the important sequence is contained in the shorter fragment.

The essential character of Tyr is obvious since the Phe-analog is inactive. It is interesting that almost every potent opioid carries a phenolic OH. This might suggest that the phenolic hydroxyl of opioids interacts with the same complementary group on the receptor surface. Not unexpectedly, substitution with two iodines ortho to the phenolic hydroxyl group resulted in an inactive compound, thus excluding the possibility of using this peptide as a radioactive ligand in receptor studies.

The low activity of the Ala derivatives points to the importance of the peptide backbone connecting the aromatic amino acids. Also the Phe residue is rather critical since Tyr in position 4 reduces the affinity by 97 %.

Very recently, Bradbury, Smyth and Snell (8), applying empirical rules, proposed that the four N-terminal amino acids of Met-enkephalin form a β-bend when the peptide interacts with the receptor. If our analogs are considered from the point of view of compatibility with β-bend formation, those with a receptor affinity above 10 appear to assume such a conformation as easily as the parent compounds. Using the empirical rules of Chou and Fasman (9), peptide V thus can be envisaged to form a β-bend, probably stabilized by a hydrogen bond involving Gly in position four, with a flexible C-terminal dipeptide tail. Most of the analogs with relative receptor affinity below 5 would give β-bends with too low stability. For peptides

VII, IX and X no explanations in those terms can yet be given.

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