Our approach to the modeling of β-endorphin has been based on the proposal that three basic structural units can be distinguished in the natural peptide hormone: a highly specific opiate recognition sequence at the N terminus (residues 1–5) connected via a hydrophilic link (residues 6–12) to a potential amphiphilic helix in the C-terminal residues 13–31. Our previous studies showed the validity of this approach and have demonstrated the importance of the amphiphilic helical structure in the C terminus of β-endorphin. The present model, peptide 5, has been designed in order to evaluate further the requirements of the amphiphilic secondary structure as well as to determine the importance of this basic structural element as compared to more specific structural features which might occur in the C-terminal segment. For these reasons, peptide 5 retains the three structural units previously postulated for β-endorphin; the major difference with regard to previous models is that the whole C-terminal segment, residues 13–31, has been built using only D-amino acids.

In aqueous buffered solutions as well as in 2,2,2-trifluoroethanol-containing solutions, the CD spectra of peptide 5 show the presence of a considerable amount of left-handed helical structure. Enzymatic degradation studies employing rat brain homogenate homogenate indicate that peptide 5 is stable in this milieu. In β- and μ-opiate receptor-binding assays, peptide 5 shows a slightly higher affinity than β-endorphin for both receptors while retaining the same β/μ selectivity. In opiate assays on the guinea pig ileum, the potency of peptide 5 is twice that of β-endorphin. In the rat vas deferens assay, which is very specific for β-endorphin, peptide 5 displays mixed agonist-antagonist activity. Most remarkably, peptide 5 displays a potent opiate analgesic effect when injected intracerebroventricularly into mice. At equal doses, the analgesic effect of peptide 5 is less than that of β-endorphin (10–15%) but longer lasting. In conjunction with our previous model studies, these results clearly demonstrate that the amphiphilic helical structure in the C terminus of β-endorphin is of predominant importance with regard to activity in rat vas deferens and analgesic assays. The similarity between the in vitro and in vivo opiate activities of β-endorphin and peptide 5, when compared to the drastic change in chirality in the latter model, demonstrates that even a left-handed amphiphilic helix formed by D-amino acids can function satisfactorily as a structural unit in a β-endorphin-like peptide.

It has been proposed that peptides which bind to amphipathic surfaces such as phospholipid vesicles, membranes or receptors, will themselves possess regions of amphiphilic secondary structure complementary to those of the target surfaces (2–8). One of these particular secondary structures, the amphiphilic helix, has been examined in several studies (4, 8–11) where synthetic peptides, a priori designed to form such a structure, have provided considerable information on the relationship between the general characteristics of the helical structure and the particular physical and biological properties of the respective peptides. In examining various peptide sequences suitable for a similar structural approach, we focused our attention on β-endorphin, a 31-residue peptide hormone with potent opiate activities (Fig. 1). We have proposed (1) that three separate regions can be distinguished in the natural sequence: a highly specific opiate recognition site in the N-terminal residues 1–5, identical to Met-enkephalin; a hydrophilic spacer region in residues 6–12; and a 16-residue sequence between Pro-13 and Gly-30 capable of forming an amphiphilic α- or π-helix (Fig. 2). The hydrophobic domain resulting from the formation of the helical structure covers one-half of the helix surface and, in the α-helical conformation, is continuous and twists along the length of the helical axis.

In order to investigate our hypothesis, four peptide models of β-endorphin were synthesized (Fig. 1) and their physical and biological properties were determined (12–14). All four peptides were able to reproduce many of the properties of β-endorphin. When differences were noticed among the properties of the model peptides, they could be rationalized on the basis of the presence of an amphiphilic helical segment in the C terminus of the natural molecule. Our results led to the conclusion that the C terminus of β-endorphin does not have a highly specific function in binding to the μ- and δ-opiate receptors or in the activity on the GPI.

Concerning the properties which are related to the potential amphiphilic segment, the presence and the shape of a hydrophobic domain strongly influence: (a) the propensity toward

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1The abbreviations used are: GPI, guinea pig ileum; RVD, rat vas deferens; [3H]DADL, [3-5-3H]Tyr[D-Ala²,D-Leu⁵]enkephalin; [3H]DHM, [1,7,8-3H]dihydromorphine; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; CD, circular dichroism; TFE, 2,2,2-trifluoroethanol; icv, intracerebroventricularly.
The formation of a helical structure as well as its inherent stability; (b) the self-association properties of the peptides; and (c) their resistance toward enzymatic degradation. We have also demonstrated, with the study of peptide 4, that the amphiphilic character of the helical region in residues 13-31 is of critical importance in the specific interaction of β-endorphin with the c-receptor (14).

It is not clear why peptide 3 is 15-30% as potent an analgesic agent as β-endorphin (13), while peptides 1 and 2 show no measurable analgesic potency. One of the possibilities is that a π-helix is the conformation required to induce an antinociceptive effect. Another possibility is that the twisted hydrophobic domain of an α-helical form is required for analgesic activity. On both helix surfaces, additional specific side chain interactions, for example an aromatic residue in position 27, may also be important.

We have now investigated the properties of a new compound, peptide 5. For this model, the natural sequence of β-endorphin was retained in residues 1-12 (Fig. 1), but the whole 13-31 segment was built using only D-amino acids and should, in a left-handed α-helical conformation, form an amphiphilic structure fairly closely related to the one found in β-endorphin or peptide 3 (Fig. 3). The results reported here fully confirm our hypotheses and, in relation to our previous studies (4-14), demonstrate the versatility as well as the usefulness of our general structural approach in the understanding of the physical and pharmacological properties of a variety of biologically active peptides.

**EXPERIMENTAL PROCEDURES**

Materials—[3H]DADL was obtained from New England Nuclear (43.6 Ci/mmol) and [3H]DHM was obtained from Amersham (65.0 Ci/mmol). Human β-endorphin was purchased from Beckman and was homogeneous by reverse phase HPLC. [D-Ala², D-Leu⁵]enkephalin and naloxone were purchased from Sigma. Morphine sulfate was obtained from Merck.

Peptide Synthesis and Purification—The solvents and reagents used for the synthesis were purified according to standard published methods (15). Boc derivatives of the amino acids employed were as follows: L- and D-glutamine, glycine, D-leucine, N²-chlorobenzyloxycarbonyl-β-tyrosine, L-Threonine, and D-lysine, L-methionine, L- and D-phenylalanine, D-proline, O-benzyl-L-serine, O-benzyl-L-threonine, and O-2,6-dichlorobenzyl-L-tyrosine.

Chloromethylated, 1% cross-linked, styrene-divinylbenzene co-polymer (0.67 mmol Cl/g) was esterified using Boc-D-glutamine and anhydrous KF (17). Hydrophobic residues are circled.

**FIG. 1.** Amino acid sequences of human β-endorphin and peptides 1 and 5. Undlined residues correspond to D-amino acids.

**FIG. 2.** Residues 13–29 of human β-endorphin represented on an α-helical net (57) (left) and a π-helical net (right). Hydrophobic residues are circled.

**FIG. 3.** Residues 13–31 of peptides 1, 3, 4, and 5, represented on an α-helical net (57). Hydrophobic residues are circled.
The peptide was then extracted from the peptide and resin mixture with 50 and 50% aqueous acetic acid containing 5% diithreitol and the extracts were lyophilized. This material was gel-filtered through a Bio-Gel P-2 column (1% AcOH, 1 mM diithreitol), and the fractions eluted in less than 2 times the void volume were pooled and lyophilized. This peptide mixture was then subjected to ion exchange chromatography on CM-Sephadex C-25 (50 mM KCl, 50 mM sodium borate, pH 8.3, containing 20% formamide) using a linear gradient of 0.15 - 0.7 M NaCl. By monitoring the absorbance at 270 nm, a major peak eluting in the middle of the gradient was collected and desalted on a Bio-Gel P-2 column (1% AcOH, 1 mM diithreitol). Corresponding fractions of peptide 5 was achieved by reverse phase HPLC on an Altex C18 semi-preparative column. The desalted solution was concentrated and loaded onto the column in portions of 1 - 2 ml. After washing out the solvents with a 0.02 M sodium phosphate buffer, 0.1 M sodium perchlorate, pH 2.6, containing 20% acetonitrile, a gradient of 40 to 45% CH3CN in the same buffer was applied over 22 min at a flow rate of 5.0 ml/min. Peptide 5 was eluted near two-thirds of the gradient and a baseline separation was achieved from the other impurities present. After desalting (Bio-Gel P-4, 1% AcOH) of the material collected by HPLC and lyophilization, peptide 5 was obtained with a high purity. Analytical HPLC on an Altex C8 column eluting at 1.5 ml/min with a gradient of 58 - 45% CH3CN in 0.02 M sodium phosphate buffer (0.1 M sodium perchlorate, pH 2.6) showed a major symmetrical peak at 210 nm with no detectable impurities. The overall yield was 3% based on the anticipated residues.

Circular Dichroism Studies—CD spectra of solutions of peptide 5 in 0.02 M sodium phosphate buffer, pH 7.4, containing 0.16 M KCl, or in mixtures of the same buffered salt solution and TFE were measured from 200 to 250 nm using a Cary 60 spectropolarimeter.

Opiate Receptor Binding—The affinities of peptide 5 for brain opiate receptors were compared to those of β-endorphin by determining the ability of these peptides to inhibit the specific binding of [3H]DADL, 0.6-0.7 nM (β-receptor assay), or [3H]DHM, 0.5-0.6 nM (μ-receptor assay), to guinea pig brain whole membrane preparations. The procedure was essentially the same as previously described (13, 19).

Opiate Assays on the Guinea Pig Ileum and Rat Vas Deferen—GPI (28) and RVD (21) opiate assays were performed according to established procedures, using white female Hartley guinea pigs (400-500 g) and male Sprague-Dawley rats (250-300 g). Assays were performed essentially as described previously (12, 13). In the GPI assays, the tissues were suspended between the electrodes at 0.5 - 1.0 g tension and subjected to electrical pulses of 1.2-ms duration at 80 V and 0.1 Hz. In the RVD assays, the isolated tissues were suspended at 0.2-g tension and stimulated with electrical pulses of 0.1-ms duration at 70 W and 0.1 Hz. In GPI or RVD assays of β-endorphin, the tissue allowed for re-equilibration was kept at a minimum (2-5 min) to reduce possible enzymatic degradation. In RVD assays, tissues were much slower to respond fully to additions of peptide 5, and the time allowed for re-equilibration was frequently greater than 30 min, so no indication of reversal of the opiate effect due to degradation of the peptide was observed. Because of the mixed agonist-antagonist behavior of peptide 5 in RVD assays, its agonist dose-response curve was achieved by single-dose challenges (22-26).

Resistance to Proteolysis—The relative resistance of peptide 5 toward degradation by proteolytic enzymes endogenous to rat brain was determined by the same method as previously described (12, 13). Aliquots corresponding to the various time points were analyzed for peptide 5 by loading 100-200 μl onto an Altex C18 analytical HPLC column fitted with a guard column and previously equilibrated with 0.02 M sodium phosphate buffer, pH 2.6, 0.1 M sodium perchlorate/acetoniitride (58:42, v/v). Peptides were eluted isocratically with 42% CH3CN at a flow rate of 1.5 ml/min. The amount of peptide 5 was quantitated by integration of its absorbance peak at 210 nm, relative to standard samples in water.

Analogic Assays—β-Endorphin and peptide 5 were tested for their antinociceptive properties by the hot plate method (27). Experiments were performed as described previously (13). Peptides were injected intracerebroventricularly (28) in 5 μl of 0.5% saline solution. Naloxone was administered subcutaneously as a single dose of 30 μg in 100 μl of saline solution.

The analogic effect on each mouse was calculated at each time point after injection using the equation: % analgesia = (PL - CL)/ (60 - s - CL) x 100, where CL is mean control latency, and PL is postinjection latency.

RESULTS

Design of Peptide 5

With this new model, we decided to assess the importance of the secondary helical structure by reproducing it using a sequence of only D-amino acids. In this regard, peptide 5 has been designed to retain the natural sequence of β-endorphin in residues 1-12, but in the C-terminal segment 13-31 only D-amino acids have been used (Fig. 1). The previously studied peptide 3 (13) was used as a basis for the construction of the hydrophobic domain of peptide 5. Assuming a left-handed α-helical conformation for the 13-31 segment of peptide 5, the respective positions of the D-amino acids in the sequence have been chosen so that they will allow the formation of an amphiphilic α-helix with a hydrophobic domain fairly similar in size and in shape to that of peptide 3 (Fig. 3). The same standard set of amino acids used in previous studies was again utilized: leucines as hydrophobic residues, glutamines as neutral hydrophilic residues, lysines as basic hydrophilic residues. The respective amounts of each of these amino acids were chosen so as to provide a high helix-forming potential (29) and to retain the overall hydrophobic-hydrophilic balance of the natural compound.

Peptide Synthesis and Purification

Peptide 5 was synthesized by standard solid phase methods and was purified by gel permeation chromatography, ion exchange chromatography and reverse phase HPLC. The final product was homogeneous by analytical reverse phase HPLC, had the expected amino acid composition, and showed the correct sequence by Edman degradation.

CD Studies

The CD spectra of peptide 5 (0.02 m sodium phosphate, pH 7.4, 0.16 m KCl) definitely show the presence of a left-handed α-helical structure, with two distinct maxima at 222 and 208 nm (30) (Fig. 4). Because of the lack of consistent data in the literature on the values of the mean residue ellipticities for this particular structure (30-34), we did not think it reasonable to calculate the relative contributions of the different secondary structures. However, from the simplicity of the CD spectra obtained, we think that no major structures other than α-helix and random coil are to be found in peptide 5.

A concentration dependence of the mean residue ellipticity at 222 nm over the range 1.0 x 10^{-5} - 2.0 x 10^{-5} M is indicative of a self-associative process (35) (Fig. 4). The experimental results can be fitted by an equation describing either cooperative trimerization or tetramerization. Since, in these calculations, the experimental values for [θ]_{208} may be affected by variations in the L- and D-amino acid random coil structures, the results should be considered only as suggestive.

The effects of various proportions of TFE on 1.0 x 10^{-5} M solutions of peptide 3 and 5 were studied. The net increase of the mean residue ellipticities at 222 and 208 nm indicates an augmentation of the helical structure at higher TFE concentration, the largest change being observed between 5 and 20% TFE (Fig. 4).
**Structural Characterization of β-Endorphin**

37°C. The results are compared to those of previous models in Table II.

**GPI**—Peptide 5 was able to inhibit almost completely the electrically stimulated contractions of this tissue. This effect was totally reversed by the opiate antagonist naloxone (1-2 μM), indicating that the model peptide was acting directly on opiate receptors. The rate of response to an added dose of peptide 5 was in the range of 2-4 min, which is similar to that previously observed for either peptide 3 or β-endorphin (12, 13). Over a period as long as 30 min, no spontaneous reversal of the opiate effect was observed, indicating an apparent stability of peptide 5 toward enzymatic degradation. No antagonist effect of peptide 5 was observed on β-endorphin action; thus, the peptides were acting in an additive way. The IC₅₀ value determined for peptide 5 by probit analysis of the dose-response curves was 29.5 ± 11.8 nM, which is almost identical to the value previously reported for peptide 3 (13).

**RVD**—Compared to previous model peptides, the action of peptide 5 on this tissue was more complex. In 30% of the experiments, some sort of temporary tetanization of the muscle could be observed on the first addition of a dose of peptide 5. This effect was not prevented by naloxone, and muscles subsequently responded to the action of either peptide 5 or β-endorphin in the same way as the ones which did not show this type of behavior.

The most important difference, as compared to previous models, was that peptide 5 displayed a mixed agonist-antagonist activity. For this reason, its agonist dose-response curve has been determined by single-dose challenges, i.e. between each tested dose, the muscle was washed several times and re-equilibrated at the maximal amplitude of the electrically stimulated twitches. The dose-response curves obtained by that method were submitted to probit analysis, and the IC₅₀ was determined as 225 ± 51 nM. This opiate agonist effect of peptide 5 on the RVD was fully reversed by micromolar concentrations of naloxone (Fig. 5).

The antagonist potency of peptide 5 was assayed by testing the activity of an identical dose of β-endorphin on the same muscle pretreated or not with peptide 5. A dose-related inhibition of the action of β-endorphin could be observed (Fig. 5), and, at a concentration of 150 nM, peptide 5 was able to antagonize almost 50% of the effect of β-endorphin. The results are summarized in Table III.

**Resistance to Proteolysis**

The resistance of peptide 5 toward proteolysis by enzymes endogenous to rat brain was assayed as described under "Experimental Procedures." HPLC analysis of aliquots with-

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**Table I**

| Peptide          | Relative potencies[^a] |
|------------------|------------------------|
|                  | [3H]DADL (β-receptors) | [3H]DHM (μ-receptors) |
| β-Endorphin      | 1[^b]                  | 1[^b]                   |
| Peptide 1        | 2.2                    | 2.9                     |
| Peptide 2        | 0.6                    | 60                      |
| Peptide 3        | 11                     | 12                      |
| Peptide 4        | 0.6                    | 1.95                    |
| Peptide 5        | 1.5                    | 1.3                     |

[^a]: Potencies are relative to those determined for β-endorphin in the same assay, using the relationship: Potency = IC₅₀ (β-endorphin)/IC₅₀ (peptide).
[^b]: In the presence of 0.46 nM [3H]DADL, IC₅₀ (β-endorphin) = 31 nM.
[^c]: In the presence of 0.15 nM [3H]DHM, IC₅₀ (β-endorphin) = 58 nM.

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**Table II**

| Peptide          | GPI IC₅₀[^a] | RVD IC₅₀[^a] |
|------------------|-------------|-------------|
| β-Endorphin      | 61 ± 13 (n = 3) | 41 ± 2 (n = 3) |
| Peptide 1        | 16 ± 2 (n = 3) | 61 ± 18 (n = 4) |
| Peptide 2        | 151 ± 21 (n = 5) | 450 ± 15 (n = 3) |
| Peptide 3        | 30 ± 10 (n = 3) | 267 ± 48 (n = 4) |
| Peptide 4        | 9.8 ± 0.5 (n = 4) | 3110 ± 600 (n = 4) |
| Peptide 5        | 29.5 ± 12 (n = 5) | 225 ± 51 (n = 5) |

[^a]: Values are the mean ± S.E.
[^b]: As the maximal inhibitory effect was not attained, this value represents 50% inhibition of the starting signal. The inhibitory effect of peptide 4 on the RVD is neither blocked nor reserved by naloxone.

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**Opiate Receptor Binding**

The abilities of peptide 5 to inhibit binding of [3H]DADL or [3H]DHM to the membranes were compared to those of β-endorphin in the same assay. At the concentrations used in these experiments, [3H]DADL should label β-receptors selectively and [3H]DHM should label μ-receptors selectively (36, 37). The affinity of peptide 5 for either μ- or β-opiate receptors was almost identical to that of β-endorphin, with the same β/μ selectivity. The results for peptide 5 are compared with those previously obtained for other models in Table I.

**Opiate Activities on the Guinea Pig Ileum and Rat Vas Deferens**

The opiate activities of peptide 5 were determined on isolated GPI and RVD preparations in Krebs-Ringer solution at

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**Fig. 4.** Circular dichroism spectra of peptide 5. Mean residue ellipticities are shown. Curve a: 2 × 10⁻⁶ peptide 5; buffer: 0.02 M sodium phosphate, pH 7.4, containing 0.16 M KCl. Curve b: 6 × 10⁻⁶ M peptide 5; buffer with 20% TFE added. Curve c: construction of a hypothetical curve from poly(L-lysine) spectra (30). The inverses of sodium phosphate, pH 7.4, containing 0.16 M peptide 5; buffer with 20% TFE added. Curve c: construction of a hypothetical curve from poly(L-lysine) spectra (30). The inverses of sodium phosphate, pH 7.4, containing 0.16 M KCl.
Peptide 5 caused significant and long lasting analgesia at doses of 1, 3, 10, and 20 μg/mouse. The maximal effect was usually attained 40–60 min after injection and thereafter was slowly reversed. Subcutaneous injection of naloxone 41 min after administration of peptide 5 caused a complete reversal of the antinociceptive effect as tested 20 min later. This demonstrates that the analgesia was mediated by opiate receptors and not by other nonopiate pathways. In these naloxone-treated mice, analgesia returned 60 min later to the level of the untreated group, presumably because naloxone was cleared from the central nervous system more rapidly than peptide 5. These effects are illustrated in Fig. 6 by the results obtained for 10-μg doses of peptide 5.

In addition to its analgesic effect, peptide 5 produced a number of other opiate-like behavioral effects that have been observed for β-endorphin (38), including explosive motor behavior, Straub tail, and catalepsy. The cataleptic state persisted during the first 3 h in mice injected with 10 and 20 μg of peptide 5.

Fig. 7 compares the time courses of analgesia for a 3-μg dose of β-endorphin, peptide 5, and peptide 3. The maximal effect for β-endorphin was already observed 20 min after injection. It diminished rapidly thereafter, and almost no significant effect could be detected 60 min after injection. Peptides 5 and 3 displayed almost a parallel time course.

![Fig. 5. Opiate activities of peptide 5 on the RVD. a, inhibitory effect of a 0.8 μM dose of peptide 5 and subsequent reversal by a 3 μM dose of naloxone. b, inhibitory action of a 30 nM dose of β-endorphin. c, inhibitory effect of a 30 nM dose of β-endorphin on the same muscle as in b, previously treated with 250 nM peptide 5.](image)

![Fig. 6. Antinociceptive effect of equal doses (3 μg) of β-endorphin, peptide 5, and peptide 3. Peptides were injected icv and the analgesic effect was determined as described under “Experimental Procedures.” β-Endorphin (●); peptide 5 (▲); peptide 3 (◆); saline controls (□). Data for β-endorphin and peptide 3 are from Ref. 13. Error bars indicate the S.E.; numbers in parentheses represent the number of mice tested.](image)

![Fig. 7. Antinociceptive effect of equal doses (3 μg) of β-endorphin, peptide 5, and peptide 3. Peptides were injected icv and the analgesic effect was determined as described under “Experimental Procedures.” β-Endorphin (●); peptide 5 (▲); peptide 3 (◆); saline controls (□). Data for β-endorphin and peptide 3 are from Ref. 13. Error bars indicate the S.E.; numbers in parentheses represent the number of mice tested.](image)
Their maximal effect was attained in about 60 min and later a slow diminution of this effect occurred during the next 90 min.

The potency of peptide 5 is compared to those of \( \beta \)-endorphin and peptide 3 in Fig. 8, using the maximum effect observed for each dose of each peptide, regardless of the time after injection. This figure shows that peptide 5 has a lower potency than \( \beta \)-endorphin for producing analgesia in mice but, as opposed to peptide 3, that its efficacy is comparable to that of the natural compound. The potency of peptide 5 for inducing analgesia can be estimated as being 10 to 15% of that of \( \beta \)-endorphin.

**DISCUSSION**

Our hypothesis (1) that there are three separate structural regions in \( \beta \)-endorphin has now been investigated by studying five model peptides. Previous reports had provided ample evidence that the enkephalin segment was an absolute requirement (21, 39–41), and our studies (12–14) have shown that the proposed spacer region (residues 6–12) did not have a very specific function with regard to the examined physical and biological properties of the model peptides. Therefore, our main interest has been the modeling of the potential amphiphilic helical segment in the C terminus of \( \beta \)-endorphin, and the results of our previous studies showed that the amphiphilic helix has a specific function with regard to analgesia and its interaction with the \( \epsilon \)-opiate receptor.

The questions that we tried to address with peptide 5 are more complex. Carrying our hypothesis to its limit, if an amphiphilic helix is the major structural determinant in the C-terminal segment of \( \beta \)-endorphin, an analog designed to possess a similar feature should display some activity even if this secondary structure is formed by a D-amino acid sequence. A consequence of using amino acids of the D-configuration should be that if there is any important stereospecific requirement in the corresponding region of the natural compound, the results obtained for the analog should point it out quite clearly.

Examination of the CD spectra of peptide 5 reveals two major points: 1) in aqueous buffered salt solutions, this peptide shows a considerable amount of left-handed \( \alpha \)-helical structure which increases on addition of TFE (Fig. 4); and 2) a dependence of \([\theta]_{222}\) on peptide 5 concentration is observed between 1 \( \times \) 10\(^{-4}\) and 2 \( \times \) 10\(^{-4}\) M (Fig. 4).

Several structure-promoting agents, including TFE, have been shown to induce helical structure in \( \beta \)-endorphin (22, 42–44), and a correlation between helicity in the C terminus of \( \beta \)-endorphin and in vitro opiate activities has been reported (45, 46). The results obtained for peptide 5 demonstrate the ability of this model peptide to adopt the same preferred helical conformation as \( \beta \)-endorphin or peptide 3, although of opposite handedness, in a medium chosen to mimic to a certain extent the environment of the opiate receptor.

Self-association at high concentration has been found to various extents in almost all of our studies dealing with amphiphilic \( \alpha \)-helical peptides (4–14), and is consistent with the presence in peptide 5 of a well defined amphiphilic structure.

In both \( \delta \) - and \( \mu \)-opiate receptor binding assays, peptide 5 was as potent as \( \beta \)-endorphin and approximately 8 times less potent than peptide 3 (Table I). These results are in good agreement with the conclusions of our previous studies (14), and the small variations in binding affinities observed either for reported \( \beta \)-endorphin analogs (47) or for models as different as peptides 1, 3, 4, and 5 (Fig. 1) definitely show that the C-terminal segment of \( \beta \)-endorphin plays a nonspecific role in the binding to these types of opiate receptors.

Previous reports (39, 40, 46) as well as our own work on \( \beta \)-endorphin analogs (12, 13), in particular the study of peptide 4 (14), had led us to the conclusion that the GPI assays were relatively insensitive to changes in the C-terminal region of \( \beta \)-endorphin. The observation that peptide 5 displays the same potency as peptide 3 and is only three times less potent than peptide 4 in this assay (Table II) shows again that, in this case, a structural element like the amphiphilic \( \alpha \)-helix is not necessary in the C terminus of \( \beta \)-endorphin. Moreover, these results strongly suggest that no specific interaction of this particular region of the molecule is needed to induce an opiate effect on the GPI.

In view of the drastic change in chirality in the 13–31 segment, it is remarkable that peptide 5 displays potent activity in the RVD assay, which is very specific for \( \beta \)-endorphin (21, 40, 41). The naloxone reversibility of this agonist effect clearly demonstrates that it is mediated by opiate receptors. The agonist IC\(_{50}\) of 225 \( \pm \) 51 nM is almost identical to that obtained for peptide 3 (Table II) and such a low value definitely indicates that peptide 5 is a \( \beta \)-endorphin analog as opposed to either a morphine or enkephalin analog for which the lower values are in the range of 4000 to 5000 nM (21, 41). Further support for this fact can be derived from the antagonist activity of peptide 5 (Table III) which, to our knowledge, is the first opiate reported to display such a behavior. One likely explanation of the mixed agonist-antagonist activity of peptide 5 is that this peptide and \( \beta \)-endorphin bind competitively to the same receptor, but that their respective efficacy in turning the receptor into an active state is markedly different.

Since peptide 4 is poorly, if at all, amphiphilic, and its action on the RVD has been shown not to be mediated by the \( \epsilon \)-receptor (14), the results obtained for peptide 5 clearly demonstrate that an amphiphilic helix is a prerequisite for \( \beta \)-endorphin to interact with the \( \epsilon \)-opiate receptor.

The reported potencies (IC\(_{50}\)) of \( \beta \)-EP-1-21 (>200 nM), \( \beta \)EP-31 (>2000 nM), and shorter N-terminal fragments (>50,000 nM) (41, 43) strongly suggest that the recognition site for the \( \epsilon \)-receptor in the C terminus of \( \beta \)-endorphin does not lie in residues 24–31. The lower potency of peptide 5, when compared to \( \beta \)-endorphin or peptide 1, is in agreement with the previously proposed (13, 35, 48, 49) importance of the presence in position 18 of an aromatic moiety, which is a prominent feature on the helix surface, and could be necessary.

![Fig. 8. Antinociceptive effect of various doses of \( \beta \)-endorphin, peptide 5, and peptide 3. The maximum analgesic effect for each dose was taken regardless of the time after injection. Values were determined as described under "Experimental Procedures." \( \beta \)-Endorphin (○); peptide 5 (●); peptide 3 (×). Data for \( \beta \)-endorphin and peptide 3 are from Ref. 13. Error bars indicate the S.E.; numbers in parentheses represent the number of mice tested at each dose.](http://www.jbc.org/Downloaded from http://www.psc.org/)
in order to induce full agonist activity.

One of the most striking properties of peptide 5 is its analgesic effect when injected iv into mice (Fig. 6). The time course of analgesia is different for \( \beta \)-endorphin and peptide 5, the effect of the latter compound being very similar to that of peptide 3 (Fig. 7). One explanation for the slower onset of the analgesic effect of peptide 5 can be derived from the proteolysis experiments, where the low recovery of this peptide from rat brain homogenate could be due to nonspecific tissue binding. This same phenomenon, in the analgesic assay, could prevent the model peptide from diffusing throughout the brain as rapidly as \( \beta \)-endorphin. Presumably, the slower diminution in potency observed for peptide 5 compared to \( \beta \)-endorphin is due to the greater resistance of the model compound toward enzymatic degradation, as shown in the \textit{in vitro} proteolysis experiments. However, the apparent stability of peptide 5 in the latter experiments still contrasts with even the slow decrease in its analgesic potency. Nevertheless, the fact that peptide 5 displays a longer lasting analgesic activity than \( \beta \)-endorphin illustrates the potential utility of our structural approach in the development of stable synthetic hormones.

The analgesic potency of peptide 5 can be estimated as being 10–15% of that of \( \beta \)-endorphin. This indicates that some of the specificity of \( \beta \)-endorphin for producing analgesic effects has been lost in the design of the model peptide. As pointed out earlier (13), potential amphiphilic \( \alpha \)-helical structures are ubiquitous in the C-terminal regions of \( \beta \)-endorphin analogs that have potent analgesic activity, including all species variants that have been tested (50). The most remarkable finding that emerges from the analgesic study of peptide 5 is that this peptide, which not only is highly nonhomologous to \( \beta \)-endorphin in its C terminus, but whose whole 13–31 segment consists of D-amino acids, displays a potency almost equivalent to that of other analogs with very minor changes in the C terminus (39, 51–56). This result makes it evident that an amphiphilic \( \alpha \)-helical structure, be it formed by L- or D-amino acids, is a predominant factor with regard to the analgesic activity of \( \beta \)-endorphin. We pointed out earlier (13) that, on the helix surface, similar features can be found in the various natural \( \beta \)-endorphins, for example, the presence of an aromatic residue at the C terminus of the hydrophobic domain, surrounded by basic residues. The lower analgesic potency observed for peptide 5, as compared to that of \( \beta \)-endorphin, could be due to a decreased ability of a left-handed helical structure to accommodate properly some of these features.

In conclusion, the study of peptide 5, as part of our general structural approach, has shown how, with a small number of synthetic peptides, it has been possible to examine thoroughly the structure-function relationships in a naturally occurring peptide hormone. For \( \beta \)-endorphin, this has been done by considering the natural polypeptide in terms of three structural domains with different particular characteristics. Combining specific design for each different model with the study of several physical and pharmacological properties having various degrees of specificity for \( \beta \)-endorphin, it has been possible to determine the different contributions of the particular domains to the overall activity profile of the natural molecule. The similarity between the \textit{in vitro} and the \textit{in vivo} opiate activities of peptide 5 and \( \beta \)-endorphin, when compared to the nonhomology in amino acid composition and most importantly to the drastic change in amino acid chirality, is a striking proof that, in biologically active peptides of moderate length, the structural factors are not to be overlooked. In this view, the structural principles outlined here and in our previous studies should have many applications in the understanding of other peptide hormones and in the development of stable synthetic biologically active peptides with a high specificity of action.

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